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(54) **Title:** CHIMERIC PROSTATE-HOMING PEPTIDES WITH PRO-APOPTOTIC ACTIVITY

(57) **Abstract:** The present invention provides a chimeric prostate-homing peptide with pro-apoptotic activity. In a preferred embodiment, the chimeric prostate-homing pro-apoptotic peptide contains the sequence SMSIARL-GG-D(KLAKLAK)₂. Methods of using such chimeric peptides for treating patients having prostate cancer also are provided.

CHIMERIC PROSTATE-HOMING PEPTIDES WITH
PRO-APOPTOTIC ACTIVITY

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

10   The present invention relates generally to the
fields of cancer biology and drug delivery and, more
specifically, to the selective targeting of antimicrobial
peptides to the prostate.

BACKGROUND INFORMATION

15   Prostatic adenocarcinoma recently became the
most frequent cancer diagnosed in American men,
surpassing the frequency of lung cancer for the first
time. An estimated 30,000 American men die annually of
this malignancy. Furthermore, prostate cancer affects
20 men throughout the world, with the highest frequencies
reported in the United States and Scandinavian countries
and a moderately high frequency seen throughout Europe.
Prostate cancer is a disease of elderly men, and of all
patients with this disease, 75% are 60 to 80 years of
25 age. At the age of 50 years, the estimated lifetime
probability of developing clinically apparent prostatic
carcinoma is about 10% for American men. However,
autopsy studies have shown that the true frequency of

2

prostatic carcinoma is actually considerably higher than
is indicated by clinical evidence.

       A major hurdle to advances in treating cancers
such as cancer of the prostate is the relative lack of
5 agents that can selectively target the cancer, while
sparing normal tissue. For example, radiation therapy
and surgery, which generally are localized treatments,
can cause substantial damage to normal tissue in the
treatment field, resulting in scarring and, in severe
10 cases, loss of function of the normal tissue.
Chemotherapy, which generally is administered
systemically, can cause substantial damage to organs such
as bone marrow, mucosae, skin and the small intestine,
which undergo rapid cell turnover and continuous cell
15 division. As a result, undesirable side effects, for
example, nausea, hair loss and reduced blood cell counts,
occur as a result of systemically treating a cancer
patient with chemotherapeutic agents. Such undesirable
side effects often limit the amount of a treatment that
20 can be administered. Due to such shortcomings in
treatment, cancer remains a leading cause of patient
morbidity and death.

       Potent antimicrobial activity has been observed
for a class of peptides including naturally occurring
25 peptides such as melittin, the gramicidins, magainins,
defensins and cecropins. Naturally occurring
antimicrobial peptides, and related synthetic
antimicrobial sequences, generally have an equivalent
number of polar and nonpolar residues within an
30 amphipathic domain and a sufficient number of basic
residues to give the peptide an overall positive charge
at neutral pH. The biological activity of amphipathic
α-helical peptides against Gram-positive bacteria may

result from the ability of these peptides to form ion
channels through membrane bilayers. Many antimicrobial
peptides selectively inhibit and kill bacteria while
maintaining low mammalian cell cytotoxicity, with the
5 differential sensitivity of bacterial cells apparently
due to membrane differences between bacteria and
mammalian cells. As shown herein, these antimicrobial
peptides can be endowed with selective cytotoxic activity
against a particular eukaryotic cell type, such as the
10 endothelium of the prostate gland.

Thus, there is a need for novel anti-cancer
therapeutics that can selectively target the prostate.
The present invention satisfies this need by providing
prostate-homing pro-apoptotic peptides that combine an
15 antimicrobial peptide with a prostate-homing peptide to
produce a conjugate with selective toxicity against the
prostate. Related advantages are provided as well.

SUMMARY OF THE INVENTION

The present invention provides a chimeric
20 prostate-homing pro-apoptotic peptide that contains a
prostate-homing peptide linked to an antimicrobial
peptide, where the chimeric peptide is selectively
internalized by prostate tissue and exhibits high
toxicity thereto, while the antimicrobial peptide has low
25 mammalian cell toxicity when not linked to the
prostate-homing peptide. In a chimeric peptide of the
invention, the prostate-homing peptide portion can
contain, for example, the sequence SMSIARL (SEQ ID NO:
207) or a functionally equivalent sequence, and the
30 antimicrobial peptide portion can have an amphipathic
α-helical structure such as the sequence $(KLAKLAK)_2$ (SEQ
ID NO: 200), $(KLAKKLA)_2$ (SEQ ID NO: 201), $(KAAKKAA)_2$ (SEQ

4

ID NO: 202) or (KLGKKLG)$_3$ (SEQ ID NO: 203). In a
preferred embodiment, the antimicrobial peptide portion
contains the sequence $_D$(KLAKLAK)$_2$. An exemplary
prostate-homing pro-apoptotic peptide is provided herein
5   as SMSIARL-GG-$_D$(KLAKLAK)$_2$. The present invention further
provides a method of directing an antimicrobial peptide
to a prostate cancer *in vivo*. The method includes the
step of administering a chimeric prostate-homing
pro-apoptotic peptide that contains a prostate-homing
10 peptide linked to an antimicrobial peptide, where the
chimeric peptide is selectively internalized by prostate
tissue and exhibits high toxicity thereto, while the
antimicrobial peptide has low mammalian cell toxicity
when not linked to the prostate-homing peptide. In a
15 method of the invention, the prostate-homing peptide can
contain, for example, the sequence SMSIARL (SEQ ID NO:
207) or a functionally equivalent sequence, and the
antimicrobial peptide can contain a sequence such as
$_D$(KLAKLAK)$_2$. In a preferred embodiment, the chimeric
20 prostate-homing pro-apoptotic peptide includes the
sequence SMSIARL-GG-$_D$(KLAKLAK)$_2$.

Also provided by the invention is a method of
inducing selective toxicity in a prostate cancer *in vivo*.
The method includes the step of administering to a
25 subject having prostate cancer a chimeric prostate-homing
pro-apoptotic peptide that contains a prostate-homing
peptide linked to an antimicrobial peptide, where the
chimeric peptide is selectively internalized by prostate
tissue and exhibits high toxicity thereto, while the
30 antimicrobial peptide has low mammalian cell toxicity
when not linked to the prostate-homing peptide. The
method of inducing selective toxicity in a prostate
cancer *in vivo* can be practiced, for example, with a
prostate-homing peptide containing the sequence SMSIARL

5

(SEQ ID NO: 207) or a functionally equivalent sequence.
The antimicrobial peptide can include, for example, the
sequence $_D$(KLAKLAK)$_2$. In a preferred embodiment, the
chimeric prostate-homing pro-apoptotic peptide includes
5 the sequence SMSIARL-GG-$_D$(KLAKLAK)$_2$.

         In addition, the invention provides a method of
treating a patient having prostate cancer by
administering to the patient a chimeric prostate-homing
pro-apoptotic peptide of the invention, whereby the
10 chimeric peptide is selectively toxic to the tumor. The
chimeric peptide contains a prostate-homing peptide
linked to an antimicrobial peptide, and the chimeric
peptide is selectively internalized by prostate tissue
and exhibits high toxicity thereto, while the
15 antimicrobial peptide has low mammalian cell toxicity
when not linked to the prostate-homing peptide. The
prostate-homing peptide portion can contain, for example,
the sequence SMSIARL (SEQ ID NO: 207) or a functionally
equivalent sequence, and the antimicrobial peptide
20 portion can contain, for example, the sequence
$_D$(KLAKLAK)$_2$. In a preferred embodiment, the chimeric
peptide contains the sequence SMSIARL-GG-$_D$(KLAKLAK)$_2$.

             BRIEF DESCRIPTION OF THE DRAWINGS

         Figure 1 shows a computer-generated model and
25 amino acid sequence of CNGRC-GG-$_D$(KLAKLAK)$_2$, designated
"HPP-1."
         Upper panel: CNGRC-GG-$_D$(KLAKLAK)$_2$ (HPP-1) is
composed of a homing domain and a membrane-disrupting
domain joined by a coupling domain.

6

Lower panel: Amino acid sequence of "HPP-1" corresponding to the structure shown in upper panel.

Figure 2 shows mitochondrial swelling and mitochondria-dependent apoptosis in the presence of
5 $_D$(KLAKLAK)$_2$.

a. Mitochondrial swelling curve (optical absorbance spectrum) is shown in the presence of $_D$(KLAKLAK)$_2$ or Ca^{+2} (positive control).

b. Immunoblot of caspase-3 cleavage showing
10 the 32 kDa proform and 8 and 20 kDa processed forms in the presence of $_D$(KLAKLAK)$_2$ or DLSLARLATARLAI (SEQ ID NO: 204) in the presence or absence of mitochondria. Typical experiments are shown. Results were reproduced in three independent experiments.

15   Figure 3 shows mitochondrial swelling and apoptosis in dermal microvessel endothelial cells treated with CNGRC-GG-$_D$(KLAKLAK)$_2$ (HPP-1).

a. Dermal microvessel endothelial cell cord formation, scale bar = 250 μm.
20   b. DEVD-pNA hydrolysis (caspase activation) in proliferating dermal microvessel endothelial cells treated with CNGRC-GG-$_D$(KLAKLAK)$_2$ (HPP-1).

c. Viability of proliferating dermal microvessel endothelial cells treated with HPP-1 (black
25 bars) or control peptide $_D$(KLAKLAK)$_2$ (gray bars) over time. (t test, $P < 0.05$).

d. Viability of cord-forming dermal microvessel endothelial cells treated with HPP-1 (black bars) or control peptide $_D$(KLAKLAK)$_2$ (gray bars) over time.
30 (t test, $P < 0.05$).

Figure 4 shows the effect of HPP-1 treatment of nude mice bearing human MDA-MB-435-derived breast carcinoma xenografts.

a. Tumor volume of HPP-1 treated tumors as compared to control CARAC-GG-$_D$(KLAKLAK)$_2$ treated tumors. Differences in tumor volumes between day 1 and day 50 are shown (t test, P=0.027).

b. Kaplan-Meier survival plot showing the survival of nude mice bearing human MDA-MB-435-derived breast carcinoma xenografts treated with HPP-1 or control peptide (mixture of $_D$(KLAKLAK)$_2$ and CNGRC (SEQ ID NO: 8). Each group was comprised of thirteen animals. (Log-Rank Test, P < 0.05).

Figure 5 shows the effect of CDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ on oxygen-induced retinal neovascularization in newborn mice. Retinal neovessel number is shown for treatments with vehicle (black bar); CDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ (striped bar); and a control mixture of unconjugated CDCRGDCFC (SEQ ID NO: 1) and $_D$(KLAKLAK)$_2$ (hatched bar).

Figure 6 shows accumulation of intravenously injected biotin conjugate of prostate-homing peptide in prostate tissue. a. Avidin-peroxidase staining of a prostate section from a mouse injected with biotin-labeled prostate homing peptide, SMSIARL (SEQ ID NO: 207). b. Avidin-peroxidase staining of a prostate section from a mouse injected with biotin-labeled control peptide CARAC (SEQ ID NO: 208).

Figure 7 shows apoptosis induced by SMSIARL-GG-$_D$(KLAKLAK)$_2$ in the normal mouse prostate. a. TUNEL staining of prostate tissue from a mouse treated with SMSIARL-GG-$_D$(KLAKLAK)$_2$ chimeric peptide. b. Larger

8

magnification of a field similar to that in a. c. TUNEL
staining of negative control mice treated with 250 μg of
an unconjugated mixture of SMSIARL (SEQ ID NO: 207) and
$_D$(KLAKLAK)$_2$.

5   Figure 8 shows survival of TRAMP mice treated
with SMSIARL-GG-$_D$(KLAKLAK)$_2$, vehicle alone, $_D$(KLAKLAK)$_2$
peptide alone, or SMSIARL peptide (SEQ ID NO: 207) alone.

           Figure 9 shows binding of prostate-homing
SMSIARL (SEQ ID NO: 207) phage to human prostate
10   vasculature.   a and b. Peroxidase staining of human
prostate tissue section containing both normal and
cancerous tissue overlaid with 10^9 TU SMSIARL phage (SEQ
ID NO: 207) and detected with anti-phage antibody. a is
an overview (x 20) while b shows a detail from panel a at
15   a higher magnification (x 40).   c. Peroxidase staining as
in panel a with phage lacking a peptide insert.   d.
Peroxidase staining as in a with soluble SMSIARL peptide
SEQ ID NO: 207 included in the overlay.

           DETAILED DESCRIPTION OF THE INVENTION

20   Antimicrobial peptides, also known as lytic
peptides or channel-forming peptides, are broad spectrum
anti-bacterial agents. These peptides typically disrupt
bacterial cell membranes, causing cell lysis and death.
Over 100 antimicrobial peptides occur naturally. In
25   addition, analogs have been synthesized de novo as
described in Javadpour et al., J. Med. Chem. 39:3107-3113
(1996); and Blondelle and Houghten, Biochem. 31:
12688-12694 (1992), each of which is incorporated herein
by reference. While some antimicrobial peptides such as
30   melittin are not selective and damage normal mammalian
cells at the minimum bactericidal concentration, others

are selective for bacterial cells. For example, the
naturally occurring magainins and cecropins exhibit
substantial bactericidal activity at concentrations that
are not lethal to normal mammalian cells.

5   Antimicrobial peptides frequently contain
cationic amino acids, which are attracted to the head
groups of anionic phospholipids, leading to the
preferential disruption of negatively charged membranes.
Once electrostatically bound, the amphipathic helices can
10 distort the lipid matrix, resulting in loss of membrane
barrier function (Epand, _The Amphipathic Helix_ CRC Press:
Boca Raton (1993); Lugtenberg and van Alphen, _Biochim._
Biophys. Acta 737:51-115 (1983), each of which is
incorporated herein by reference). Prokaryotic
15 cytoplasmic membranes maintain large transmembrane
potentials and have a high content of anionic
phospholipids. In contrast, the outer leaflet of
eukaryotic plasma membranes generally has low, or no,
membrane potential and is almost exclusively composed of
20 zwitterionic phospholipids. Thus, due to distinct
membrane compositions, antimicrobial peptides can
preferentially disrupt prokaryotic membranes as compared
to eukaryotic membranes.

            The present invention is directed to the
25 surprising discovery that an antimicrobial peptide
sequence can be linked to a tumor homing molecule to
produce a homing pro-apoptotic conjugate that generally
is non-toxic outside of eukaryotic cells but which
promotes disruption of mitochondrial membranes and
30 subsequent cell death when targeted and internalized by
eukaryotic cells. Homing pro-apoptotic conjugates such
as HPP-1, which contains the antimicrobial peptide
$_D$(KLAKLAK)$_2$ linked to the cyclic tumor homing molecule

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CNGRC (SEQ ID NO: 8), can have selective toxicity against
angiogenic endothelial cells *in vivo* and, thus, be useful
as a new class of anti-cancer therapeutics.

5   Thus, the present invention provides a homing
pro-apoptotic conjugate, which includes a tumor homing
molecule that selectively homes to a selected mammalian
cell type or tissue linked to an antimicrobial peptide,
where the conjugate is selectively internalized by the
mammalian cell type or tissue and exhibits high toxicity
10 thereto, and where the antimicrobial peptide has low
mammalian cell toxicity when not linked to the tumor
homing molecule. For example, a homing pro-apoptotic
conjugate of the invention can exhibit selective toxicity
against angiogenic endothelial cells and can be useful,
15 for example, in methods of inducing selective toxicity *in
vivo* in a tumor having angiogenic vasculature.

        As disclosed herein, a synthetic antimicrobial
peptide with selective toxicity against bacteria as
compared to eukaryotic cells, $_D$(KLAKLAK)$_2$, induced marked
20 mitochondrial swelling at a concentration of 10 µM
(Figure 2a), significantly less than the concentration
required to kill eukaryotic cells, indicating that
$_D$(KLAKLAK)$_2$ preferentially disrupts mitochondrial
membranes as compared to eukaryotic membranes (see
25 Example I). Moreover, $_D$(KLAKLAK)$_2$ activated
mitochondria-dependent cell-free apoptosis as measured by
characteristic caspase-3 processing (Figure 2b) while a
non-α-helix forming peptide DLSLARLATARLAI (SEQ ID
NO: 204) did not. These results indicate that
30 antimicrobial peptides such as $_D$(KLAKLAK)$_2$ can disrupt
mitochondrial membranes, which, like bacterial membranes,
have a high content of anionic phospholipids, reflecting
the common ancestry of bacteria and mitochondria (Epand,

supra, 1993; Lugtenberg and van Alphen, *supra,* 1983;
Matsuzaki et al., <u>Biochemistry</u> 34:6521-6526 (1995);
Hovius et al., <u>FEBS Lett.</u> 330:71-76 (1993); and
Baltcheffsky and Baltcheffsky in Lee et al., <u>Mitochondria</u>
5   <u>and Microsomes</u> Addison-Wesley: Reading, MA (1981), each
of which is incorporated herein by reference).

As further disclosed herein, the antimicrobial
peptide $_D$(KLAKLAK)$_2$ was conjugated to the cyclic tumor
homing peptide CNGRC (SEQ ID NO: 8) via a glycinylglycine
10 bridge to produce the peptide CNGRC-GG-$_D$(KLAKLAK)$_2$,
designated "HPP-1." As disclosed herein, HPP-1 was
tested in a tissue culture model of angiogenesis by
assaying cord formation, which is a form of migration
indicated by a change in endothelial cell morphology from
15 the usual "cobblestones" to chains or cords of cells.
Treatment of normal human dermal microvessel cells
(DMECs) with 60 μM HPP-1 led to a decrease in percent
viability with time under the conditions of proliferation
(Figure 3c) or cord formation (Figure 3d), while
20 treatment with untargeted $_D$(KLAKLAK)$_2$ peptide led to only
a negligible loss in viability (see Example II).
Furthermore, as shown in Table 1, the LC$_{50}$ for
proliferating or migrating DMECs treated with HPP-1 was
an order of magnitude lower than the LC$_{50}$ for angiostatic
25 DMECs maintained in a monolayer at 100% confluency,
demonstrating preferential killing by HPP-1 under
angiogenic conditions. The results disclosed herein
further demonstrate that the mitochondria of DMECs
treated for 24 hours with $_D$(KLAKLAK)$_2$ remained
30 morphologically normal, while those treated with
CNGRC-GG-$_D$(KLAKLAK)$_2$ or ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ displayed
altered mitochondrial morphology before exhibiting the
classical morphological indicators of apoptosis including
nuclear condensation and fragmentation.

12

As shown in Example III, the HPP-1 peptide
CNGRC-GG-$_D$(KLAKLAK)$_2$ also has activity *in vivo*. As
disclosed in Figures 4a and b, nude mice bearing human
MDA-MD-435 breast carcinoma xenografts were treated with
5   HPP-1. Tumor volume was smaller on average by one order
of magnitude, and survival longer in the HPP-1 treated
animal groups as compared to control groups.
Furthermore, some of the HPP-1 treated mice outlived
control mice by several months, indicating that both
10 primary tumor growth and metastasis were inhibited.
Destruction of tumor architecture and widespread cell
death was evident upon histopathological analysis of the
tumors, with about 50% apoptotic cell death. HPP-1 also
was effective against tumors derived from the human
15 melanoma cell line C8161; and ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$
was effective against MDA-MD-435 breast carcinoma tumors.
In sum, these results indicate that homing pro-apoptotic
peptides based on tumor homing and antimicrobial peptide
sequences can be non-toxic outside of eukaryotic cells
20 but can promote disruption of mitochondrial membranes and
subsequent cell death when internalized by the targeted
eukaryotic target cells. Homing pro-apoptotic peptides
such as HPP-1, which have selective toxicity against
angiogenic endothelial cells, can be particularly
25 valuable as anti-cancer therapeutics.

Further results disclosed herein show that
retinal neovascularization can be selectively inhibited
by a homing pro-apoptotic conjugate of the invention. In
particular, the number of retinal neovessels in mice
30 treated with the homing pro-apoptotic conjugate
CDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ was reduced to 30-40% of control
levels (see Figure 5). Thus, a homing pro-apoptotic
conjugate of the invention can contain a tumor homing
molecule, or can contain another homing molecule that

13

selectively homes to a selected mammalian cell type or tissue.

     A homing pro-apoptotic conjugate of the invention is characterized by being highly toxic to the mammalian cell type in which it is internalized. As used herein, the term "highly toxic" means that the conjugate is relatively effective in resulting in cell death of a selected cell type or tissue. One skilled in the art understands that toxicity can be analyzed using one of a variety of well known assays for cell viability. In general, the term highly toxic is used to refer to a conjugate in which the concentration for half maximal killing (LC_{50}) is less than about 100 µM, preferably less than about 50 µM. For example, as disclosed herein, the homing pro-apoptotic conjugate HPP-1 was characterized by LC_{50}s of 51, 34 and 42, respectively, for angiogenic proliferating and cord forming DMEM cells and for KS1767 cells. Moreover, the prolonged survival of tumor-bearing mice treating with a homing pro-apoptotic conjugate of the invention demonstrates that the selective toxicity can be reproduced *in vivo*.

     As used herein, the term "antimicrobial peptide" means a naturally occurring or synthetic peptide having antimicrobial activity, which is the ability to kill or slow the growth of one or more microbes. An antimicrobial peptide can, for example, kill or slow the growth of one or more strains of bacteria including a Gram-positive or Gram-negative bacteria, or a fungi or protozoa. Thus, an antimicrobial peptide can have, for example, bacteriostatic or bacteriocidal activity against, for example, one or more strains of *Escherichia coli, Pseudomonas aeruginosa* or *Staphylococcus aureus*. While not wishing to be bound by the following, an

14

antimicrobial peptide can have biological activity due to
the ability to form ion channels through membrane
bilayers as a consequence of self-aggregation.

5   An antimicrobial peptide is typically highly
basic and can have a linear or cyclic structure. As
discussed further below, an antimicrobial peptide can
have an amphipathic α-helical structure (see U.S. Patent
5,789,542; Javadpour et al., *supra,* 1996; Blondelle and
Houghten, *supra,* 1992). An antimicrobial peptide also
10 can be, for example, a β-strand/sheet-forming peptide as
described in Mancheno et al., J. Peptide Res. 51:142-148
(1998).

       An antimicrobial peptide can be a naturally
occurring or synthetic peptide. Naturally occurring
15 antimicrobial peptides have been isolated from biological
sources such as bacteria, insects, amphibians and mammals
and are thought to represent inducible defense proteins
that can protect the host organism from bacterial
infection. Naturally occurring antimicrobial peptides
20 include the gramicidins, magainins, mellitins, defensins
and cecropins (see, for example, Maloy and Kari,
Biopolymers 37:105-122 (1995); Alvarez-Bravo et al.,
Biochem. J. 302:535-538 (1994); Bessalle et al., FEBS
274:151-155 (1990); and Blondelle and Houghten in Bristol
25 (Ed.), Annual Reports in Medicinal Chemistry pages
159-168 Academic Press, San Diego, each of which is
herein incorporated by reference). As discussed further
below, an antimicrobial peptide also can be an analog of
a natural peptide, especially one that retains or
30 enhances amphipathicity.

15

An antimicrobial peptide incorporated within a homing pro-apoptotic conjugate of the invention has low mammalian cell toxicity when not linked to a tumor homing molecule. Mammalian cell toxicity readily can be
5 assessed using routine assays. For example, mammalian cell toxicity can be assayed by lysis of human erythrocytes *in vitro* as described in Javadpour et al., *supra,* 1996. An antimicrobial peptide having "low mammalian cell toxicity" is not lytic to human
10 erythrocytes or requires concentrations of greater than 100 µM for lytic activity, preferably concentrations greater than 200, 300, 500 or 1000 µM.

In a preferred embodiment, the invention also provides a homing pro-apoptotic conjugate in which the
15 antimicrobial peptide portion promotes disruption of mitochondrial membranes when internalized by eukaryotic cells. In particular, such an antimicrobial peptide preferentially disrupts mitochondrial membranes as compared to eukaryotic membranes. Mitochondrial
20 membranes, like bacterial membranes but in contrast to eukaryotic plasma membranes, have a high content of negatively charged phospholipids. An antimicrobial peptide can be assayed for activity in disrupting mitochondrial membranes using, for example, an assay for
25 mitochondrial swelling (as described in Example I) or another assay well known in the art. As disclosed herein, for example, $_D$(KLAKLAK)$_2$ induced marked mitochondrial swelling at a concentration of 10 µM, significantly less than the concentration required to
30 kill eukaryotic cells. An antimicrobial peptide that induces significant mitochondrial swelling at, for example, 50 µM, 40 µM, 30 µM, 20 µM, 10 µM, or less, is considered a peptide that promotes disruption of mitochondrial membranes.

16

        The invention also provides a homing
pro-apoptotic conjugate in which a tumor homing molecule
is linked to an antimicrobial peptide having an
amphipathic α-helical structure. In a homing
5   pro-apoptotic conjugate of the invention, the
antimicrobial peptide portion can have, for example, the
sequence (KLAKLAK)$_2$ (SEQ ID NO: 200); (KLAKKLA)$_2$ (SEQ ID
NO: 201);(KAAKKAA)$_2$ (SEQ ID NO: 202); or (KLGKKLG)$_3$ (SEQ
ID NO: 203), in particular the sequence $_D$(KLAKLAK)$_2$. A
10 homing pro-apoptotic conjugate of the invention can have,
for example, the sequence CNGRC-GG-$_D$(KLAKLAK)$_2$ or
ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$.

        Antimicrobial peptides generally have random
15 coil conformations in dilute aqueous solutions, yet high
levels of helicity can be induced by helix-promoting
solvents and amphipathic media such as micelles,
synthetic bilayers or cell membranes. α-Helical
structures are well known in the art, with an ideal
20 α-helix characterized by having 3.6 residues per turn and
a translation of 1.5 Å per residue (5.4Å per turn; see
Creighton, _Proteins: Structures and Molecular Properties_
W.H Freeman, New York (1984)). In an amphipathic
α-helical structure, polar and non-polar amino acid
25 residues are aligned into an amphipathic helix, which is
an α-helix in which the hydrophobic amino acid residues
are predominantly on one face, with hydrophilic residues
predominantly on the opposite face when the peptide is
viewed along the helical axis.

30   Antimicrobial peptides of widely varying
sequence have been isolated, sharing an amphipathic
α-helical structure as a common feature (Saberwal et al.,
Biochim. Biophys. Acta 1197:109-131 (1994)). Analogs of
native peptides with amino acid substitutions predicted

17

to enhance amphipathicity and helicity typically have
increased antimicrobial activity. In general, analogs
with increased antimicrobial activity also have increased
cytotoxicity against mammalian cells (Maloy et al.,
5 Biopolymers 37:105-122 (1995)).

          As used herein in reference to an antimicrobial
peptide, the term "amphipathic α-helical structure" means
an α-helix with a hydrophilic face containing several
polar residues at physiological pH and a hydrophobic face
10 containing nonpolar residues. A polar residue can be,
for example, a lysine or arginine residue, while a
nonpolar residue can be, for example, a leucine or
alanine residue. An antimicrobial peptide having an
amphipathic α-helical structure generally has an
15 equivalent number of polar and nonpolar residues within
the amphipathic domain and a sufficient number of basic
residues to give the peptide an overall positive charge
at neutral pH (Saberwal et al., Biochim. Biophys. Acta
1197:109-131 (1994), which is incorporated by reference
20 herein). One skilled in the art understands that
helix-promoting amino acids such as leucine and alanine
can be advantageously included in an antimicrobial
peptide of the invention (see, for example, Creighton,
supra, 1984).

25   A variety of antimicrobial peptides having an
amphipathic α-helical structure are well known in the
art. Such peptides include synthetic, minimalist
peptides based on a heptad building block scheme in which
repetitive heptads are composed of repetitive trimers
30 with an additional residue. Such synthetic antimicrobial
peptides include, for example, peptides of the general
formula $[(X_1X_2X_2)(X_1X_2X_2)X_1]_n$ (SEQ ID NO: 205) or
$[(X_1X_2X_2)X_1(X_1X_2X_2)]_n$ (SEQ ID NO: 206), where X_1 is a polar

18

residue, X_2 is a nonpolar residue; and n is 2 or 3 (see
Javadpour et al., *supra,* 1996. (KLAKLAK)$_2$ (SEQ ID
NO: 200); (KLAKKLA)$_2$ (SEQ ID NO: 201); (KAAKKAA)$_2$ (SEQ ID
NO: 202); and (KLGKKLG)$_3$ (SEQ ID NO: 203) are examples of
5 synthetic antimicrobial peptides having an amphipathic
α-helical structure. Similar synthetic, antimicrobial
peptides having an amphipathic α-helical structure also
are known in the art, for example, as described in U.S.
Patent No. 5,789,542 to McLaughlin and Becker.

10   '   Helicity readily can be determined by one
skilled in the art, for example, using circular dichroism
spectroscopy. Percent α-helicity can be determined, for
example, after measuring molar ellipticity at 222 nm as
described in Javadpour et al., *supra,* 1996 (see, also,
15 McLean et al., <u>Biochemistry</u> 30:31-37 (1991), which is
incorporated by reference herein). An amphipathic
α-helical antimicrobial peptide of the invention can
have, for example, at least about 20% helicity when
assayed in amphipathic media such as 25 mM SDS. One
20 skilled in the art understands that such an antimicrobial
peptide having an amphipathic α-helical structure can
have, for example, at least about 25%, 30%, 35% or 40%
helicity when assayed in 25 mM SDS. An antimicrobial
peptide having an α-helical structure can have, for
25 example, from 25% to 90% helicity; 25% to 60% helicity;
25% to 50% helicity; 25% to 40% helicity; 30% to 90%
helicity; 30% to 60% helicity; 30% to 50% helicity; 40%
to 90% helicity or 40% to 60% helicity when in assayed in
25 mM SDS. Amphipathicity can readily be determined, for
30 example, using a helical wheel representation of the
peptide (see, for example, Blondelle and Houghten, *supra,*
1994).

   The structure of an exemplary homing
pro-apoptotic conjugate of the invention,
CNGRC-GG-$_D$(KLAKLAK)$_2$, is illustrated in Figure 1. As can
be seen in Figure 1, the homing domain, CNGRC (SEQ ID
5 NO: 8) is a disulfide-bonded cyclic structure and is
coupled to a membrane disrupting domain, $_D$(KLAKLAKKLAKLAK)
via a glycinylglycine bridge. The D-amino acids in the
membrane disrupting, antimicrobial portion of the
conjugate can be useful in imparting increased stability
10 upon the conjugate in vivo. Furthermore, the membrane
disrupting $_D$(KLAKLAKKLAKLAK) portion forms an amphipathic
helix. In particular, the lysine residues are aligned on
one face of the helix (shown as dark shaded region of
helix), while the non-polar leucine and alanine residues
15 are aligned on the opposite (light-shaded) face of the
helix.

   A homing pro-apoptotic conjugate of the
invention can be a chimeric peptide in which the tumor
homing molecule is a tumor homing peptide. A homing
20 pro-apoptotic chimeric peptide of the invention can have
a variety of lengths, from about 18 amino acids to about
fifty amino acids or more. A chimeric peptide of the
invention can have, for example, from about 20 to about
fifty amino acids, preferably from 20 to 40 amino acids,
25 more preferably from 20 to 30 amino acids. Such a
chimeric peptide can have, for example, an upper length
of 40, 35, 30, 27, 25 or 21 amino acids. A chimeric
peptide of the invention can be linear or cyclic. In a
preferred embodiment, a homing pro-apoptotic chimeric
30 peptide of the invention includes a cyclic tumor homing
peptide portion.

20

A homing pro-apoptotic chimeric peptide of the invention also can be a peptidomimetic. As used herein, the term "peptidomimetic" is used broadly to mean a peptide-like molecule that has substantially the activity
5 of the corresponding peptide. Peptidomimetics include chemically modified peptides, peptide-like molecules containing non-naturally occurring amino acids, peptoids and the like, have the selective homing activity and the high toxicity of the peptide from which the
10 peptidomimetic is derived (see, for example, "Burger's Medicinal Chemistry and Drug Discovery" 5th ed., vols. 1 to 3 (ed. M.E. Wolff; Wiley Interscience 1995), which is incorporated herein by reference). For example, D amino acids can be advantageously included in the antimicrobial
15 peptide portion of a chimeric peptide of the invention (see Examples I and II). Peptidomimetics provide various advantages over a peptide, including increased stability during passage through the digestive tract and, therefore, can be advantageously used as oral
20 therapeutics.

In a homing pro-apoptotic conjugate of the invention, a "coupling domain" can be used to link a tumor homing peptide and an antimicrobial peptide and can, for example, impart flexibility to the conjugate as
25 a whole. A coupling domain can be, for example, a glycinylglycine linker, alaninylalanine linker or other linker incorporating glycine, alanine or other amino acids. The use of a glycinylglycine coupling domain is described in Example II.
30

The vasculature within a tumor generally undergoes active angiogenesis, resulting in the continual formation of new blood vessels to support the growing tumor. Such angiogenic blood vessels are distinguishable

from mature vasculature in that angiogenic vasculature
expresses unique endothelial cell surface markers,
including the $\alpha_v\beta_3$ integrin (Brooks, <u>Cell</u> 79:1157-1164
(1994); WO 95/14714, Int. Filing Date November 22, 1994)

5   and receptors for angiogenic growth factors (Mustonen and
Alitalo, <u>J. Cell Biol.</u> 129:895-898 (1995); Lappi, <u>Semin.</u>
<u>Cancer Biol.</u> 6:279-288 (1995)). Moreover, tumor
vasculature is histologically distinguishable from other
blood vessels in that tumor vasculature is fenestrated

10 (Folkman, <u>Nature Med.</u> 1:27-31 (1995); Rak et al.,
<u>Anticancer Drugs</u> 6:3-18 (1995)). Thus, the unique
characteristics of tumor vasculature make it a
particularly attractive target for anti-cancer
therapeutics.

15   As disclosed herein, tumor homing molecules can
bind to the endothelial lining of small blood vessels of
tumors. The vasculature within tumors is distinct,
presumably due to the continual neovascularization,
resulting in the formation of new blood vessels required

20 for tumor growth. The distinct properties of the
angiogenic neovasculature within tumors are reflected in
the presence of specific markers in endothelial cells and
pericytes (Folkman, <u>Nature Biotechnol.</u> 15:510 (1997);
Risau, <u>FASEB J.</u> 9:926-933 (1995); Brooks et al., *supra*,

25 1994); these markers likely are being targeted by the
disclosed tumor homing molecules.

            The ability of a tumor homing molecule to
target the blood vessels in a tumor provides substantial
advantages over methods of systemic treatment or methods

30 that directly target the tumor cells. For example, tumor
cells depend on a vascular supply for survival and the
endothelial lining of blood vessels is readily accessible
to a circulating probe. Conversely, in order to reach

22

solid tumor cells, a therapeutic agent must overcome
potentially long diffusion distances, closely packed
tumor cells, and a dense fibrous stroma with a high
interstitial pressure that impedes extravasation (Burrows
5   and Thorpe, Pharmacol. Ther. 64:155-174 (1994)).

In addition, where the tumor vasculature is
targeted, the killing of all target cells may not be
required, since partial denudation of the endothelium can
lead to the formation of an occlusive thrombus halting
10 the blood flow through the entirety of the affected tumor
vessel (Burrows and Thorpe, *supra*, 1994). Furthermore,
unlike direct tumor targeting, there is an intrinsic
amplification mechanism in tumor vasculature targeting.
A single capillary loop can supply nutrients to up to
15 100 tumor cells, each of which is critically dependent on
the blood supply (Denekamp, Cancer Metast. Rev. 9:267-282
(1990); Folkman, *supra*, 1997).

As set forth above and exemplified herein, a
tumor homing molecule that is selective for the
20 angiogenic endothelial cells of tumor vasculature can be
particularly useful for directing a pro-apoptotic
antimicrobial peptide to tumor vasculature, while
reducing the likelihood that the pro-apoptotic
antimicrobial peptide will have a toxic effect on normal,
25 healthy organs or tissues. Thus, in one embodiment, the
invention provides a homing pro-apoptotic conjugate,
which includes a tumor homing molecule that selectively
homes to angiogenic endothelial cells linked to an
antimicrobial peptide, where the conjugate is selectively
30 internalized by angiogenic endothelial cells and exhibits
high toxicity thereto, and where the antimicrobial
peptide has low mammalian cell toxicity when not linked
to the tumor homing molecule.

As used herein, the term "selective toxicity" means enhanced cell death in a selected cell type or tissue as compared to a control cell type or tissue. In general, selective toxicity is characterized by at least
5 a two-fold greater extent of cell death in the selected cell type or tissue, such as angiogenic endothelial cells, as compared to a control cell type or tissue, for example, angiostatic endothelial cells. Thus, as used herein, the term selective toxicity encompasses specific
10 toxicity, whereby cell death occurs essentially only the selected cell type or tissue, as well as toxicity occurring in a limited number of cell types or tissues in addition to the selected cell type or tissue. One skilled in the art further understands that the term
15 selective toxicity refers to cell death effected by all mechanisms including apoptotic and necrotic cell death. Thus, a homing pro-apoptotic conjugate of the invention that exhibits selective toxicity for angiogenic endothelial cells effects enhanced cell death of the
20 angiogenic endothelial cells as compared to angiostatic endothelial cells or surrounding cells of other types.

As disclosed herein, identified tumor homing molecules are useful for targeting a desired antimicrobial peptide, which is linked to the homing
25 molecule, to a selected cell type such as angiogenic endothelial cells. After being internalized by the angiogenic endothelial cells in tumor vasculature, the antimicrobial peptide is toxic to the endothelial cells, thereby restricting the blood supply to the tumor and
30 inhibiting tumor growth.

24

A tumor homing molecule useful in the homing
pro-apoptotic conjugates of the invention can be a
peptide containing, for example, an NGR motif, such as
CNGRC (SEQ ID NO: 8); NGRAHA (SEQ ID NO: 6) or
5   CNGRCVSGCAGRC (SEQ ID NO: 3). A tumor homing molecule
useful in the invention also can contain an RGD motif and
can be, for example, CDCRGDCFC (SEQ ID NO: 1), or can
contain a GSL motif, such as the peptide CGSLVRC (SEQ ID
NO: 5). Additional tumor homing molecules can be
10 identified by screening a library of molecules by *in vivo*
panning as set forth in further detail below (see, also,
Examples IV to VIII; United States Patent No. 5,622,699,
issued April 22, 1997; and Pasqualini and Ruoslahti,
Nature 380:364-366 (1996), each of which is incorporated
15 herein by reference).

The term "tumor homing molecule," as used
herein, means an organic chemical such as a drug; a
nucleic acid molecule; a peptide or peptidomimetic or
protein that selectively homes *in vivo* to a selected cell
20 type or tissue. By "selectively homes" is meant that, *in
vivo*, the tumor homing molecule binds preferentially to a
selected cell type or tissue as compared to a control
cell type, tissue or organ and generally is characterized
by at least a two-fold greater localization at the
25 selected cell type or tissue compared to a control cell
type or tissue. A tumor homing molecule useful in the
invention can be, for example, a molecule that binds
preferentially to the endothelial cells of angiogenic
vasculature as compared to other cell types or
30 angiostatic vasculature.

Tumor homing molecules were identified using *in vivo* panning as follows. By panning *in vivo* against a breast carcinoma, a melanoma and a Kaposi's sarcoma, phage expressing various peptides that selectively homed to tumors were identified (see Tables 2, 3 and 4, respectively). Due to the large size of the phage (900-1000 nm) and the short time the phage were allowed to circulate (3 to 5 min), it is unlikely that a substantial number of phage would have exited the circulatory system, particularly in the brain and kidney. Tissue staining studies indicated that the tumor homing molecules that were identified primarily homed to and bound endothelial cell surface markers, which likely are expressed in an organ-specific manner. These results indicate that *in vivo* panning can be used to identify and analyze endothelial cell specificities. Such an analysis is not possible using endothelial cells in culture because the cultured cells tend to lose their tissue-specific differences (Pauli and Lee, Lab. Invest. 58:379-387 (1988)).

Although the conditions under which the *in vivo* pannings were performed identified tumor homing peptides that generally bind to endothelial cell markers, the specific presence of phage expressing tumor homing peptides also was observed in tumor parenchyma, particularly at later times after administration of the peptides (Example VII). These results demonstrate that phage expressing peptides can pass through the blood vessels in the tumor, possibly due to the fenestrated nature of the blood vessels, and indicate that the *in vivo* panning method can be useful for identifying target molecules expressed by tumor cells, as well as target molecules expressed by endothelial cells.

Phage peptide display libraries were
constructed essentially as described in Smith and Scott
(*supra*, 1993; see, also, Koivunen et al., <u>Biotechnology</u>
13:265-270 (1995); Koivunen et al., <u>Meth. Enzymol.</u>
5   245:346-369 (1994b), each of which is incorporated herein
by reference). Oligonucleotides encoding peptides having
substantially random amino acid sequences were
synthesized based on an "NNK" codon, wherein "N" is A, T,
C or G and "K" is G or T. "NNK" encodes 32 triplets,
10 which encode the twenty amino acids and an amber STOP
codon (Scott and Smith, *supra*, 1990). In some libraries,
at least one codon encoding cysteine also was included in
each oligonucleotide so that cyclic peptides could be
formed through disulfide linkages (Example IV). The
15 oligonucleotides were inserted in frame with the sequence
encoding the gene III protein (gIII) in the vector fuse 5
such that a peptide-gIII fusion protein was expressed.
Following expression, the fusion protein was expressed on
the surface of the phage containing the vector (Koivunen
20 et al., *supra*, 1994b; Smith and Scott, *supra*, 1993).

Following *in vivo* panning, the phage isolated
based on their ability to selectively home to human
breast carcinoma, mouse melanoma or human Kaposi's
sarcoma tumors displayed only a few different peptide
25 sequences (see Tables 2, 3 and 4, respectively). One of
the screenings revealed peptide sequences that contained
the arginine-glycine-aspartic acid (RGD) integrin
recognition sequence (Ruoslahti, <u>Ann. Rev. Cell Devel.</u>
<u>Biol.</u> 12:697 (1996)) in the context of a peptide
30 previously demonstrated to bind selectively to
α_v-containing integrins (Koivunen et al., *supra*, 1995;
WO 95/14714). The sequences of most of the remaining
tumor homing peptides did not reveal any significant
similarities with known ligands for endothelial cell

27

receptors. However, one of the tumor homing peptides
contained the asparagine-glycine-arginine (NGR) motif,
which is a weak integrin binding motif similar to the
motifs present in integrin-binding peptides (Ruoslahti et
5 al., U.S. Patent No. 5,536,814, issued July 16, 1996,
which is incorporated herein by reference; see, also,
Koivunen et al., *supra*, 1994a). Other screenings have
revealed numerous NGR-containing peptides (see Table 2).
Despite the weak integrin binding ability of NGR
10 peptides, an integrin receptor may not be the target
molecule recognized by the NGR tumor homing peptides
exemplified herein. As used herein, the term "integrin"
means a heterodimeric cell surface adhesion receptor.

            The peptides expressed by the phage that homed
15 to the breast tumor included the peptides CGRECPRLCQSSC
(SEQ ID NO: 2) and CNGRCVSGCAGRC (SEQ ID NO: 3; see
Table 2; Example V). Similarly, tumor homing peptides,
including the peptides CDCRGDCFC (SEQ ID NO: 1) and
CGSLVRC (SEQ ID NO: 5), were identified from two other
20 phage libraries administered to breast tumor bearing mice
(Table 2). Some of these motifs, as well as novel ones,
also were isolated by screening against mouse melanoma
and human Kaposi's sarcoma (see Tables 3 and 4). These
results demonstrated that tumor homing molecules can be
25 identified using *in vivo* panning.

            Three main tumor homing motifs that were
identified can be particularly useful in the homing
pro-apoptotic conjugates of the invention. Homing
pro-apoptotic conjugates in which the tumor homing
30 molecule portion contains an NGR motif, RGD motif or GSL
motif, can be used to target a linked antimicrobial
peptide to the endothelial cells of angiogenic
vasculature.

28

In one embodiment, the invention provides a
homing pro-apoptotic conjugate, which includes a tumor
homing peptide containing the sequence NGR linked to an
antimicrobial peptide. In such a homing pro-apoptotic
5   conjugate of the invention, the tumor homing peptide can
be, for example, CNGRC (SEQ ID NO: 8); NGRAHA (SEQ ID
NO: 6) or CNGRCVSGCAGRC (SEQ ID NO: 3). In a preferred
embodiment, the homing pro-apoptotic conjugate includes
the sequence CNGRC-GG-$_D$(KLAKLAK)$_2$.

10   In another embodiment, the invention provides a
homing pro-apoptotic conjugate, which includes a tumor
homing peptide containing the sequence RGD linked to an
antimicrobial peptide. In such a homing pro-apoptotic
conjugate, the tumor homing peptide can be, for example,
15 CDCRGDCFC (SEQ ID NO: 1). In a preferred embodiment, the
homing pro-apoptotic conjugate includes the sequence
ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$.

The invention additionally provides a homing
pro-apoptotic conjugate, which includes a tumor homing
20 peptide containing the sequence GSL linked to an
antimicrobial peptide. In such a homing pro-apoptotic
conjugate, the tumor homing peptide can be, for example,
CGSLVRC (SEQ ID NO: 5).

As discussed above, one motif contained the
25 sequence RGD (Ruoslahti, *supra*, 1996) embedded in the
peptide structure, CDCRGDCFC (SEQ ID NO: 1), which is
known to bind selectively to α_v integrins (Koivunen et
al., *supra*, 1995; WO 95/14714). Since the $\alpha_v\beta_3$ and $\alpha_v\beta_5$
integrins are markers of angiogenic vessels (Brooks et
30 al., *supra*, 1994; Friedlander et al., Science 270:1500
(1995)), a phage expressing the peptide CDCRGDCFC (SEQ ID
NO: 1) was examined for tumor targeting and, as disclosed

29

herein, homed to tumors in a highly selective manner (see
Example VI). Furthermore, homing by the CDCRGDCFC (SEQ
ID NO: 1) phage was inhibited by coadministration of the
free CDCRGDCFC (SEQ ID NO: 1) peptide.

5   Another breast tumor homing peptide had the
sequence CNGRCVSGCAGRC (SEQ ID NO: 3), which contains the
NGR motif previously shown to have weak integrin binding
activity (Koivunen et al., *J. Biol. Chem.* 268:20205-20210
(1993); Koivunen et al., *supra*, 1994a; WO 95/14714).
10 Since an NGR containing peptide was identified, two
additional peptides, the linear peptide, NGRAHA (SEQ ID
NO: 6), and the cyclic peptide, CVLNGRMEC (SEQ ID NO: 7),
each of which contains the NGR motif, were examined for
tumor homing. Like the phage expressing CNGRCVSGCAGRC
15 (SEQ ID NO: 3), phage expressing NGRAHA (SEQ ID NO: 6) or
CVLNGRMEC (SEQ ID NO: 7) homed to the tumors.
Furthermore, tumor homing was not dependent on the tumor
type or on species, as the phage accumulated selectively
in human breast carcinoma, as well as in the tumors of
20 mice bearing a mouse melanoma and mice bearing a human
Kaposi's sarcoma xenograft.

           The various peptides, including RGD- and
NGR-containing peptides, generally were bound to the
tumor blood vessels. The minimal cyclic NGR peptide,
25 CNGRC (SEQ ID NO: 8), was synthesized based on the
CNGRCVSGCAGRC (SEQ ID NO: 3) sequence. When the CNGRC
(SEQ ID NO: 8) peptide was co-injected with phage
expressing either CNGRCVSGCAGRC (SEQ ID NO: 3), NGRAHA
(SEQ ID NO: 6) or CVLNGRMEC (SEQ ID NO: 7), accumulation
30 of the phage in the breast carcinoma xenografts was
inhibited. However, the CNGRC (SEQ ID NO: 8) peptide did
not inhibit the homing of phage expressing the CDCRGDCFC
(SEQ ID NO: 1) peptide, even when administered in amounts

up to ten times higher than those that inhibited the
homing of the NGR phage. In comparison, the CDCRGDCFC
(SEQ ID NO: 1) peptide partially inhibited the homing of
the NGR phage, although the amount needed was 5 to
5 10 fold higher than that of the CNGRC peptide (SEQ ID
NO: 8). These results indicate that NGR peptides and RGD
peptides bind to different receptor sites in tumor
vasculature.

        A third motif, GSL (glycine-serine-leucine),
10 also was identified following *in vivo* panning in mice
bearing breast carcinoma, malignant melanoma or Kaposi's
sarcoma. Homing of phage expressing the GSL peptide,
CGSLVRC (SEQ ID NO: 5), was inhibited by coadministration
of the free CGSLVRC (SEQ ID NO: 5) peptide. Like the RGD
15 and NGR peptides, phage expressing GSL peptides also
bound to blood vessels of tumors. In view of the
identification of the conserved RGD, NGR and GSL motifs
present in tumor homing peptides, as disclosed herein, it
will be recognized that peptides containing such motifs
20 can be useful as tumor homing peptides and, in
particular, for forming homing pro-apoptotic conjugates
that can selectively deliver an antimicrobial peptide to
a tumor.

        Various peptide libraries containing up to
25 13 amino acids were constructed, and the NGR peptide,
CNGRCVSGCAGRC (SEQ ID NO: 3), was obtained as a result of
in vivo panning against a breast tumor. This NGR
peptide, which was obtained by screening a random peptide
library, was a tumor homing peptide. In addition, when a
30 peptide library was constructed based on the formula
CXXXNGRXX (SEQ ID NO: 13) or CXXCNGRCX (SEQ ID NO: 14),
each of which is biased toward NGR sequences, and used

for *in vivo* panning against a breast tumor, numerous NGR
peptides were obtained (see Table 2).

These results indicate that a tumor homing
molecule of the invention can comprise the amino acid
5   sequence RGD or NGR or GSL. Such a tumor homing molecule
can be a peptide as small as five amino acids, such as
CNGRC (SEQ ID NO: 8). Such tumor homing peptides also
can be not only at least 13 amino acids in length, which
is the largest peptide exemplified herein, but can be up
10 to 20 amino acids, or 30 amino acids, or 50 to 100 amino
acids in length, as desired. A tumor homing peptide of
the invention conveniently is produced by chemical
synthesis.

Immunohistochemical analysis was performed by
15 comparing tissue staining for phage allowed to circulate
for about four minutes, followed by perfusion through the
heart of the mice, or with tissues analyzed 24 hours
after phage injection. At 24 hours following
administration, essentially no phage remain in the
20 circulation and, therefore, perfusion is not required
(Pasqualini et al., *supra*, 1997). Strong phage staining
was observed in tumor vasculature, but not in normal
endothelium, in samples examined four minutes after
administration of the CNGRCVSGCAGRC (SEQ ID NO: 3) phage
25 (Example VII). In comparison, staining of the tumor was
strong at 24 hours and appeared to have spread outside
the blood vessels into the tumor parenchyma. The NGRAHA
(SEQ ID NO: 6) and CVLNGRMEC (SEQ ID NO: 7) phage showed
similar staining patterns (Example VII). In contrast,
30 the control organs and tissues showed little or no
immunostaining, confirming the specificity of the NGR
motifs for tumor vessels. Spleen and liver, however,
captured phage, as expected, since uptake by the

reticuloendothelial system is a general property of phage
particles, independent of the presence of peptide
expression by the phage (Pasqualini et al., *supra*, 1997).

5   Immunostaining also was observed following
administration of phage expressing the GSL motif
containing peptide, CLSGSLSC (SEQ ID NO: 4), and, like
that of the NGR peptides, was localized to the blood
vessels, in this case, within a melanoma tumor (see
below; see, also, Examples VII and VIII). Similarly,
10   immunostaining following administration of phage
expressing the RGD motif containing peptide, CDCRGDCFC
(SEQ ID NO: 1), to breast tumor bearing mice was
localized to the blood vessels in the tumor, but was not
observed in brain, kidney or various other nontumor
15   tissues (see Examples VI and VII; see, also, Pasqualini
et al., *supra*, 1997). These results demonstrate that the
various tumor homing peptides generally home to tumor
vasculature.

        The general applicability of the *in vivo*
20   panning method for identifying tumor homing molecules was
examined by injecting mice bearing a syngeneic melanoma
with phage expressing a diverse population of peptides
(Example VIII). The B16 mouse melanoma model was
selected for these studies because the tumors that form
25   are highly vascularized and because the biology of this
tumor line has been thoroughly characterized (see Miner
et al., <u>Cancer Res.</u> 42:4631-4638 (1982)). Furthermore,
because the B16 melanoma cells are of mouse origin,
species differences between the host and the tumor cell
30   donor will not affect, for example, the distribution of
phage into the tumor as compared to into normal organs.
As disclosed herein, *in vivo* panning against B16 melanoma
cells revealed tumor homing peptides, including, for

example, the GSL moiety containing peptide CLSGSLSC (SEQ
ID NO: 4; see, also, Table 3) and immunohistochemical
staining of the tumor and other organs using an
anti-phage antibody demonstrated that the CLSGSLSC (SEQ
5   ID NO: 4) expressing phage resulted in immunostaining in
the melanoma, but essentially no staining in skin, kidney
or other control organs (Example VIII). The staining
pattern generally followed the blood vessels within the
melanoma, but was not strictly confined to the blood
10 vessels.

Although *in vivo* panning was performed in mice,
tumor homing molecules such as peptides comprising an
NGR, RGD or GSL motif also likely can target human
vasculature. The NGR phage binds to blood vessels in the
15 transplanted human breast tumor, but not to blood vessels
in normal tissues, indicating that this motif can be
particularly useful for tumor targeting in patients. The
CDCRGDCFC (SEQ ID NO: 1) peptide binds to human
α_v-integrins (Koivunen et al., *supra*, 1995), which are
20 selectively expressed in tumor blood vessels of human
patients (Max et al., <u>Int. J. Cancer</u> 71:320 (1997); Max
et al., <u>Int. J. Cancer</u> 72:706 (1997)). Use of a homing
pro-apoptotic conjugate in which CDCRGDCFC (SEQ ID NO: 1)
is linked to an antimicrobial peptide provides the
25 additional advantage that the antimicrobial peptide can
be targeted to tumor cells, themselves, because breast
carcinoma cells, for example, can express the $\alpha_v\beta_3$
integrin (Pasqualini et al., *supra*, 1997). In fact, many
human tumors express this integrin, which may be involved
30 in the progression of certain tumors such as malignant
melanomas (Albelda et al., <u>Cancer Res.</u> 50:6757-6764
(1990); Danen et al., <u>Int. J. Cancer</u> 61:491-496 (1995);
Felding-Habermann et al., <u>J. Clin. Invest.</u> 89:2018-2022
(1992); Sanders et al., <u>Cold Spring Harb. Symp. Quant.</u>

34

Biol. 58:233-240 (1992); Mitjans et al., J. Cell. Sci.
108:3067-3078 (1995)). Unlike the CDCRGDCFC (SEQ ID
NO: 1) peptide, the NGR peptides do not appear to bind to
MDA-MD-435 breast carcinoma cells. However, NGR peptides
5   were able to deliver a therapeutically effective amount
of doxorubicin to breast tumors, indicating that, even
where a tumor homing molecule homes only to tumor
vasculature, i.e., not directly to the tumor cells, such
vasculature targeting in sufficient to confer the effect
10 of the moiety linked to the molecule.

Since the $\alpha_v\beta_3$ integrin is expressed by
endothelial cells in angiogenic vasculature, experiments
were performed to determine whether tumor vasculature
that is undergoing angiogenesis can be targeted in vivo
15 using methods as disclosed herein. Phage expressing the
peptide, CDCRGDCFC (SEQ ID NO: 1; see, Koivunen et al.,
supra, 1995), which is known to bind to the $\alpha_v\beta_3$ integrin,
were injected into mice bearing tumors formed from human
breast carcinoma cells, mouse melanoma cells or human
20 Kaposi's sarcoma cells (see Example VII). The CDCRGDCFC
(SEQ ID NO: 1) phage selectively homed to each of the
tumors, whereas such homing did not occur with control
phage. For example, in mice bearing tumors formed by
implantation of human breast carcinoma cells, a twenty-
25 to eighty-fold greater number of the CDCRGDCFC (SEQ ID
NO: 1) phage, as compared to unselected control phage,
accumulated in the tumor.

Tissue staining for the phage showed
accumulation of the CDCRGDCFC (SEQ ID NO: 1) phage in the
30 blood vessels within the tumor, whereas no staining was
observed in brain, kidney or other control organs.
Specificity of tumor homing by the CDCRGDCFC (SEQ ID
NO: 1) phage was demonstrated by competition experiments,

in which coinjection of the free CDCRGDCFC (SEQ ID NO: 1)
peptide greatly reduced tumor homing of the RGD phage,
whereas coinjection of a non-RGD-containing control
peptide had no effect on homing of the RGD phage (see
5 Example VI). These results demonstrate that the $\alpha_v\beta_3$
target molecule is expressed on the luminal surface of
endothelial cells in a tumor and that a peptide that
binds to an α_v-containing integrin can bind selectively to
this integrin and, therefore, to vasculature undergoing
10 angiogenesis.

    The results of these studies indicate that
tumor homing molecules can be identified by *in vivo*
panning and that, in some cases, a tumor homing molecule
can home to vascular tissue in the tumor as well as to
15 tumor parenchyma, probably due to the fenestrated nature
of the blood vessels permitting ready exit of the phage
from the circulatory system. Due to the ability of such
tumor homing molecules to home to tumors, the molecules
are useful for targeting a linked antimicrobial peptide
20 to tumors. Thus, the invention provides conjugates
comprising a tumor homing molecule linked to a moiety,
such conjugates being useful for targeting the moiety to
tumor cells.

    The ability of a molecule that homes to a
25 particular tumor to selectively home to another tumor of
the same or a similar histologic type can be determined
using, for example, human tumors grown in nude mice or
mouse tumors grown in syngeneic mice for these
experiments. For example, various human breast cancer
30 cell lines, including MDA-MB-435 breast carcinoma (Price
et al., Cancer Res. 50:717-721 (1990)), SKBR-1-II and
SK-BR-3 (Fogh et al., J. Natl. Cancer Inst. 59:221-226
(1975)), and mouse mammary tumor lines, including EMT6

36

(Rosen et al., _Int. J. Cancer_ 57:706-714 (1994)) and
C3-L5 (Lala and Parhar, _Int. J. Cancer_ 54:677-684
(1993)), are readily available and commonly used as
models for human breast cancer. Using such breast tumor
5 models, for example, information relating to the
specificity of an identified breast tumor homing molecule
for diverse breast tumors can be obtained and molecules
that home to a broad range of different breast tumors or
provide the most favorable specificity profiles can be
10 identified. In addition, such analyses can yield new
information, for example, about tumor stroma, since
stromal cell gene expression, like that of endothelial
cells, can be modified by the tumor in ways that cannot
be reproduced _in vitro_.

15   Selective homing of a molecule such as a
peptide or protein to a tumor can be due to specific
recognition by the peptide of a particular cell target
molecule such as a cell surface receptor present on a
cell in the tumor. Selectivity of homing is dependent on
20 the particular target molecule being expressed on only
one or a few different cell types, such that the molecule
homes primarily to the tumor. As discussed above, the
identified tumor homing peptides, at least in part, can
recognize endothelial cell surface markers in the blood
25 vessels present in the tumors. However, most cell types,
particularly cell types that are unique to an organ or
tissue, can express unique target molecules. Thus, _in
vivo_ panning can be used to identify molecules that
selectively home to a particular type of tumor cell such
30 as a breast cancer cell; specific homing can be
demonstrated by performing the appropriate competition
experiments.

37

As used herein, the term "tumor" means a mass of cells that are characterized, at least in part, by containing angiogenic vasculature. The term "tumor" is used broadly to include the tumor parenchymal cells as well as the supporting stroma, including the angiogenic blood vessels that infiltrate the tumor parenchymal cell mass. Although a tumor generally is a malignant tumor, i.e., a "cancer," a tumor also can be nonmalignant, provided that neovascularization is associated with the tumor. The term "normal" or "nontumor" tissue is used to refer to tissue that is not a "tumor." As disclosed herein, a tumor homing molecule can be identified based on its ability to home a tumor, but not to a corresponding nontumor tissue.

As used herein, the term "corresponding," when used in reference to tumors or tissues or both, means that two or more tumors, or two or more tissues, or a tumor and a tissue are of the same histologic type. The skilled artisan will recognize that the histologic type of a tissue is a function of the cells comprising the tissue. Thus, the artisan will recognize, for example, that a nontumor tissue corresponding to a breast tumor is normal breast tissue, whereas a nontumor tissue corresponding to a melanoma is skin, which contains melanocytes. Furthermore, for purposes of the invention, it is recognized that a tumor homing molecule can bind specifically to a target molecule expressed by the vasculature in a tumor, which generally contains blood vessels undergoing neovascularization, in which case a tissue corresponding to the tumor would comprise nontumor tissue containing blood vessels that are not undergoing active angiogenesis.

A tumor homing molecule useful in the
invention can be identified by screening a library of
molecules by *in vivo* panning as disclosed herein and set
forth in United States Patent No. 5,622,699, issued April
5   22, 1997; and Pasqualini and Ruoslahti, <u>Nature</u>
380:364-366 (1996), each of which is incorporated herein
by reference). As used herein, the term "library" means
a collection of molecules. A library can contain a few
or a large number of different molecules, varying from
10 about ten molecules to several billion molecules or more.
If desired, a molecule can be linked to a tag, which can
facilitate recovery or identification of the molecule.

As used herein, the term "molecule" is used
broadly to mean a polymeric or non-polymeric organic
15 chemical such as a drug; a nucleic acid molecule such as
an RNA, a cDNA or an oligonucleotide; a peptide,
including a variant or modified peptide or peptide-like
molecules, referred to herein as peptidomimetics, which
mimic the activity of a peptide; or a protein such as an
20 antibody or a growth factor receptor or a fragment
thereof such as an Fv, Fd or Fab fragment of an antibody,
which contains a binding domain. For convenience, the
term "peptide" is used broadly herein to mean peptides,
proteins, fragments of proteins and the like. A molecule
25 also can be a non-naturally occurring molecule, which
does not occur in nature, but is produced as a result of
in vitro methods, or can be a naturally occurring
molecule such as a protein or fragment thereof expressed
from a cDNA library.

30   A tumor homing molecule also can be a
peptidomimetic. As used herein, the term
"peptidomimetic" is used broadly to mean a peptide-like
molecule that has the binding activity of the tumor

homing peptide. With respect to the tumor homing
peptides of the invention, peptidomimetics, which include
chemically modified peptides, peptide-like molecules
containing non-naturally occurring amino acids, peptoids
5 and the like, have the binding activity of a tumor homing
peptide upon which the peptidomimetic is derived (see,
for example, "Burger's Medicinal Chemistry and Drug
Discovery," *supra,* 1995).

        Methods for identifying a peptidomimetic are
10 well known in the art and include, for example, the
screening of databases that contain libraries of
potential peptidomimetics. For example, the Cambridge
Structural Database contains a collection of greater than
300,000 compounds that have known crystal structures
15 (Allen et al., <u>Acta Crystallogr.</u> Section B, 35:2331
(1979)). This structural depository is continually
updated as new crystal structures are determined and can
be screened for compounds having suitable shapes, for
example, the same shape as a tumor homing molecule, as
20 well as potential geometrical and chemical
complementarity to a target molecule bound by a tumor
homing peptide. Where no crystal structure of a tumor
homing peptide or a target molecule that binds the tumor
homing molecule is available, a structure can be
25 generated using, for example, the program CONCORD
(Rusinko et al., <u>J. Chem. Inf. Comput. Sci.</u> 29:251
(1989)). Another database, the Available Chemicals
Directory (Molecular Design Limited, Informations
Systems; San Leandro CA), contains about 100,000
30 compounds that are commercially available and also can be
searched to identify potential peptidomimetics of a tumor
homing molecule.

Methods for preparing libraries containing
diverse populations of various types of molecules such as
peptides, peptoids and peptidomimetics are well known in
the art and various libraries are commercially available
5   (see, for example, Ecker and Crooke, _Biotechnology_
13:351-360 (1995), and Blondelle et al., _Trends Anal._
Chem. 14:83-92 (1995), and the references cited therein,
each of which is incorporated herein by reference; see,
also, Goodman and Ro, _Peptidomimetics for Drug Design_, in
10 "Burger's Medicinal Chemistry and Drug Discovery" Vol. 1
(ed. M.E. Wolff; John Wiley & Sons 1995), pages 803-861,
and Gordon et al., _J. Med. Chem._ 37:1385-1401 (1994),
each of which is incorporated herein by reference).
Where a molecule is a peptide, protein or fragment
15 thereof, the molecule can be produced _in vitro_ directly
or can be expressed from a nucleic acid, which can be
produced _in vitro_. Methods of synthetic peptide and
nucleic acid chemistry are well known in the art.

A library of molecules also can be produced,
20 for example, by constructing a cDNA expression library
from mRNA collected from a cell, tissue, organ or
organism of interest. Methods for producing such
libraries are well known in the art (see, for example,
Sambrook et al., _Molecular Cloning: A laboratory manual_
25 (Cold Spring Harbor Laboratory Press 1989), which is
incorporated herein by reference). Preferably, a peptide
encoded by the cDNA is expressed on the surface of a cell
or a virus containing the cDNA. For example, cDNA can be
cloned into a phage vector such as fuse 5 (Example IV),
30 wherein, upon expression, the encoded peptide is
expressed as a fusion protein on the surface of the
phage.

41

In addition, a library of molecules can
comprise a library of nucleic acid molecules, which can
be DNA or RNA or an analog thereof. Nucleic acid
molecules that bind, for example, to a cell surface
5   receptor are well known (see, for example, O'Connell et
al., *Proc. Natl. Acad. Sci., USA* 93:5883-5887 (1996);
Tuerk and Gold, *Science* 249:505-510 (1990); Gold et al.,
Ann. Rev. Biochem. 64:763-797 (1995), each of which is
incorporated herein by reference). Thus, a library of
10 nucleic acid molecules can be administered to a subject
having a tumor, and tumor homing molecules subsequently
identified by *in vivo* panning. If desired, the nucleic
acid molecules can be nucleic acid analogs that, for
example, are less susceptible to attack by nucleases
15 (see, for example, Jelinek et al., *Biochemistry*
34:11363-11372 (1995); Latham et al., *Nucl. Acids Res.*
22:2817-2822 (1994); Tam et al., *Nucl. Acids Res.*
22:977-986 (1994); Reed et al., *Cancer Res.* 59:6565-6570
(1990), each of which is incorporated herein by
20 reference).

As set forth herein, *in vivo* panning can be
used to identify a tumor homing molecule, which can be
linked to an antimicrobial peptide to form a homing
pro-apoptotic conjugate of the invention. *In vivo*
25 panning comprises administering a library to a subject,
collecting a sample of a tumor and identifying a tumor
homing molecule. The presence of a tumor homing molecule
can be identified using various methods well known in the
art. Generally, the presence of a tumor homing molecule
30 in a tumor is identified based on one or more
characteristics common to the molecules present in the
library, then the structure of a particular tumor homing
molecule is identified. For example, a highly sensitive
detection method such as mass spectrometry, either alone

or in combination with a method such as gas
chromatography, can be used to identify tumor homing
molecules in a tumor. Thus, where a library comprises
diverse molecules based generally on the structure of an
5 organic molecule such as a drug, a tumor homing molecule
can be identified by determining the presence of a parent
peak for the particular molecule.

          If desired, the tumor can be collected, then
processed using a method such as HPLC, which can provide
10 a fraction enriched in molecules having a defined range
of molecular weights or polar or nonpolar characteristics
or the like, depending, for example, on the general
characteristics of the molecules comprising the library.
Conditions for HPLC will depend on the chemistry of the
15 particular molecule and are well known to those skilled
in the art. Similarly, methods for bulk removal of
potentially interfering cellular materials such as DNA,
RNA, proteins, lipids or carbohydrates are well known in
the art, as are methods for enriching a fraction
20 containing an organic molecule using, for example,
methods of selective extraction. Where a library
comprises a population of diverse organic chemical
molecules, each linked to a specific oligonucleotide tag,
such that the specific molecule can be identified by
25 determining the oligonucleotide sequence using polymerase
chain reaction (PCR), genomic DNA can be removed from the
sample of the collected tumor in order to reduce the
potential for background PCR reactions. In addition, a
library can comprise a population of diverse molecules
30 such as organic chemical molecules, each linked to a
common, shared tag. Based on the presence and properties
of the shared tag, molecules of the library that
selectively home to a tumor can be substantially isolated
from a sample of the tumor. These and other methods can

43

be useful for enriching a sample of a collected tumor for
the particular tumor homing molecule, thereby removing
potentially contaminating materials from the collected
tumor sample and increasing the sensitivity of detecting
5   a molecule.

        Evidence provided herein indicates that a
sufficient number of tumor homing molecules selectively
homes to a tumor during *in vivo* panning such that the
molecules readily can be identified. For example,
10   various independent phage expressing the same peptide
were identified in tumors formed from implanted human
breast cancer cells (Table 2), from mouse melanoma cells
(Table 3) or from human Kaposi's sarcoma cells (Table 4).

        Although a substantial fraction of the
15   identified tumor homing molecules have the same
structure, the peptide inserts of only a small number of
isolated phage were determined. It should be recognized,
however, that hundreds of thousands to millions of phage
expressing organ homing peptides have been recovered
20   following *in vivo* pannings for organ homing molecules
(see, for example, U.S. Patent No. 5,622,699; Pasqualini
and Ruoslahti, *supra*, 1996). These results indicate that
specific tumor homing molecules will be present in
substantial numbers in a tumor following *in vivo* homing,
25   thereby increasing the ease with which the molecules can
be identified.

        Ease of identification of a tumor homing
molecule, particularly an untagged molecule, depends on
various factors, including the presence of potentially
30   contaminating background cellular material. Thus, where
the tumor homing molecule is an untagged peptide, a
larger number must home to the tumor in order to identify

44

the specific peptides against the background of cellular
protein. In contrast, a much smaller number of an
untagged organic chemical homing molecule such as a drug
is identifiable because such molecules normally are
5 absent from or present in only small numbers in the body.
In such a case, a highly sensitive method such as mass
spectrometry can be used to identify a tumor homing
molecule. The skilled artisan will recognize that the
method of identifying a molecule will depend, in part, on
10 the chemistry of the particular molecule.

       Where a tumor homing molecule is a nucleic acid
molecule or is tagged with a nucleic acid molecule, an
assay such as PCR can be particularly useful for
identifying the presence of the molecule because, in
15 principle, PCR can detect the presence of a single
nucleic acid molecule (see, for example, Erlich, PCR
Technology: Principles and Applications for DNA
Amplification (Stockton Press 1989), which is
incorporated herein by reference). Preliminary studies
20 have demonstrated that, following intravenous injection
of 10 ng of an approximately 6000 base pair plasmid into
a mouse and 2 minutes in the circulation, the plasmid was
detectable by PCR in a sample of lung. These results
indicate that nucleic acid molecules are sufficiently
25 stable when administered into the circulation such that
in vivo panning can be used to identify nucleic acid
molecules that selectively home to a tumor.

       The molecules of a library can be tagged, which
can facilitate recovery or identification of the
30 molecule. As used herein, the term "tag" means a
physical, chemical or biological moiety such as a plastic
microbead, an oligonucleotide or a bacteriophage,
respectively, that is linked to a molecule of the

library. Methods for tagging a molecule are well known
in the art (Hermanson, Bioconjugate Techniques (Academic
Press 1996), which is incorporated herein by reference).

        A tag, which can be a shared tag or a specific
5   tag, can be useful for identifying the presence or
structure of a tumor homing molecule of a library. As
used herein, the term "shared tag" means a physical,
chemical or biological moiety that is common to each
molecule in a library. Biotin, for example, can be a
10 shared tag that is linked to each molecule in a library.
A shared tag can be useful to identify the presence of a
molecule of the library in a sample and also can be
useful to substantially isolate the molecules from a
sample. For example, where the shared tag is biotin, the
15 biotin-tagged molecules in a library can be substantially
isolated by binding to streptavidin, or their presence
can be identified by binding with a labeled streptavidin.
Where a library is a phage display library, the phage
that express the peptides are another example of a shared
20 tag, since each peptide of the library is linked to a
phage. In addition, a peptide such as the hemaglutinin
antigen can be a shared tag that is linked to each
molecule in a library, thereby allowing the use of an
antibody specific for the hemaglutinin antigen to
25 substantially isolate molecules of the library from a
sample of a selected tumor.

        A shared tag also can be a nucleic acid
sequence that can be useful to identify the presence of
molecules of the library in a sample or to substantially
30 isolate molecules of a library from a sample. For
example, each of the molecules of a library can be linked
to the same selected nucleotide sequence, which
constitutes the shared tag. An affinity column

46

containing a nucleotide sequence that is complementary to
the shared tag then can be used to hybridize molecules of
the library containing the shared tag, thus substantially
isolating the molecules from a tumor sample. A

5   nucleotide sequence complementary to a portion of the
shared nucleotide sequence tag also can be used as a PCR
primer such that the presence of molecules containing the
shared tag can be identified in a sample by PCR.

A tag also can be a specific tag. As used

10 herein, the term "specific tag" means a physical,
chemical or biological tag that is linked to a particular
molecule in a library and is unique for that particular
molecule. A specific tag is particularly useful if it is
readily identifiable. A nucleotide sequence that is

15 unique for a particular molecule of a library is an
example of a specific tag. For example, the method of
synthesizing peptides tagged with a unique nucleotide
sequence provides a library of molecules, each containing
a specific tag, such that upon determining the nucleotide

20 sequence, the identity of the peptide is known (see
Brenner and Lerner, _Proc. Natl. Acad. Sci., USA_
89:5381-5383 (1992), which is incorporated herein by
reference). The use of a nucleotide sequence as a
specific tag for a peptide or other type of molecule

25 provides a simple means to identify the presence of the
molecule in a sample because an extremely sensitive
method such as PCR can be used to determine the
nucleotide sequence of the specific tag, thereby
identifying the sequence of the molecule linked thereto.

30 Similarly, the nucleic acid sequence encoding a peptide
expressed on a phage is another example of a specific
tag, since sequencing of the specific tag identifies the
amino acid sequence of the expressed peptide.

The presence of a shared tag or a specific tag can provide a means to identify or recover a tumor homing molecule following *in vivo* panning. In addition, the combination of a shared tag and specific tag can be particularly useful for identifying a tumor homing molecule. For example, a library of peptides can be prepared such that each is linked to a specific nucleotide sequence tag (see, for example, Brenner and Lerner, *supra*, 1992), wherein each specific nucleotide sequence tag has incorporated therein a shared tag such as biotin. Upon homing to a tumor, the particular tumor homing peptides can be substantially isolated from a sample of the tumor based on the shared tag and the specific peptides can be identified, for example, by PCR of the specific tag (see Erlich, *supra*, 1989).

A tag also can serve as a support. As used herein, the term "support" means a tag having a defined surface to which a molecule can be attached. In general, a tag useful as a support is a shared tag. For example, a support can be a biological tag such as a virus or virus-like particle such as a bacteriophage ("phage"); a bacterium such as *E. coli*; or a eukaryotic cell such as a yeast, insect or mammalian cell; or can be a physical tag such as a liposome or a microbead, which can be composed of a plastic, agarose, gelatin or other biological or inert material. If desired, a shared tag useful as a support can have linked thereto a specific tag. Thus, a phage display library, for example, can be considered to consist of the phage, which is a shared tag that also is a support, and the nucleic acid sequence encoding the expressed peptide, the nucleic acid sequence being a specific tag.

48

In general, a support should have a diameter
less than about 10 μm to about 50 μm in its shortest
dimension, such that the support can pass relatively
unhindered through the capillary beds present in the
5   subject and not occlude circulation. In addition, a
support can be nontoxic, so that it does not perturb the
normal expression of cell surface molecules or normal
physiology of the subject, and biodegradable,
particularly where the subject used for *in vivo* panning
10 is not sacrificed to collect a selected tumor.

Where a molecule is linked to a support, the
tagged molecule comprises the molecule attached to the
surface of the support, such that the part of the
molecule suspected of being able to interact with a
15 target molecule in a cell in the subject is positioned so
as to be able to participate in the interaction. For
example, where the tumor homing molecule is suspected of
being a ligand for a growth factor receptor, the binding
portion of the molecule attached to a support is
20 positioned so it can interact with the growth factor
receptor on a cell in the tumor. If desired, an
appropriate spacer molecule can be positioned between the
molecule and the support such that the ability of the
potential tumor homing molecule to interact with the
25 target molecule is not hindered. A spacer molecule also
can contain a reactive group, which provides a convenient
and efficient means of linking a molecule to a support
and, if desired, can contain a tag, which can facilitate
recovery or identification of the molecule (see
30 Hermanson, *supra*, 1996).

49

        As exemplified herein, a peptide suspected of
being able to home to a selected tumor such as a breast
carcinoma or a melanoma was expressed as the N-terminus
of a fusion protein, wherein the C-terminus consisted of
5   a phage coat protein. Upon expression of the fusion
protein, the C-terminal coat protein linked the fusion
protein to the surface of a phage such that the
N-terminal peptide was in a position to interact with a
target molecule in the tumor. Thus, a molecule having a
10 shared tag was formed by the linking of a peptide to a
phage, wherein the phage provided a biological support,
the peptide molecule was linked as a fusion protein, the
phage-encoded portion of the fusion protein acted as a
spacer molecule, and the nucleic acid encoding the
15 peptide provided a specific tag allowing identification
of a tumor homing peptide.

        As used herein, the term "*in vivo* panning,"
when used in reference to the identification of a tumor
homing molecule, means a method of screening a library by
20 administering the library to a subject and identifying a
molecule that selectively homes to a tumor in the subject
(see U.S. Patent No. 5,622,699). The term "administering
to a subject," when used in reference to a library of
molecules or a portion of such a library, is used in its
25 broadest sense to mean that the library is delivered to a
tumor in the subject, which, generally, is a vertebrate,
particularly a mammal such as a human.

        A library can be administered to a subject, for
example, by injecting the library into the circulation of
30 the subject such that the molecules pass through the
tumor; after an appropriate period of time, circulation
is terminated by sacrificing the subject or by removing a
sample of the tumor (Example IV; see, also, U.S. Patent

No. 5,622,699; Pasqualini and Ruoslahti, *supra*, 1996).
Alternatively, a cannula can be inserted into a blood
vessel in the subject, such that the library is
administered by perfusion for an appropriate period of
5   time, after which the library can be removed from the
circulation through the cannula or the subject can be
sacrificed to collect the tumor, or the tumor can be
sampled, to terminate circulation. Similarly, a library
can be shunted through one or a few organs, including the
10 tumor, by cannulation of the appropriate blood vessels in
the subject. It is recognized that a library also can be
administered to an isolated perfused tumor. Such panning
in an isolated perfused tumor can be useful to identify
molecules that bind to the tumor and, if desired, can be
15 used as an initial screening of a library.

        The use of *in vivo* panning to identify tumor
homing molecules is exemplified herein by screening a
phage peptide display library in tumor-bearing mice and
identifying specific peptides that selectively homed to a
20 breast tumor or to a melanoma tumor (Example IV).
However, phage libraries that display protein receptor
molecules, including, for example, an antibody or an
antigen binding fragment of an antibody such an Fv, Fd or
Fab fragment; a hormone receptor such as a growth factor
25 receptor; or a cell adhesion receptor such as an integrin
or a selectin also can be used to practice the invention.
Variants of such molecules can be constructed using well
known methods such as random mutagenesis, site-directed
mutagenesis or codon based mutagenesis (see Huse, U.S.
30 Patent No. 5,264,563, issued November 23, 1993, which is
incorporated herein by reference). If desired, peptides
can be chemically modified following expression of the
phage but prior to administration to the subject. Thus,

various types of phage display libraries can be screened
by *in vivo* panning.

Phage display technology provides a means for
expressing a diverse population of random or selectively
5   randomized peptides. Various methods of phage display
and methods for producing diverse populations of peptides
are well known in the art. For example, Ladner et al.
(U.S. Patent No. 5,223,409, issued June 29, 1993, which
is incorporated herein by reference) describe methods for
10   preparing diverse populations of binding domains on the
surface of a phage. In particular, Ladner et al.
describe phage vectors useful for producing a phage
display library, as well as methods for selecting
potential binding domains and producing randomly or
15   selectively mutated binding domains.

Similarly, Smith and Scott (<u>Meth. Enzymol.</u>
217:228-257 (1993); see, also, Scott and Smith, <u>Science</u>
249: 386-390 (1990), each of which is incorporated herein
by reference) describe methods of producing phage peptide
20   display libraries, including vectors and methods of
diversifying the population of peptides that are
expressed (see, also, Huse, WO 91/07141 and WO 91/07149,
each of which is incorporated herein by reference; see,
also, Example IV). Phage display technology can be
25   particularly powerful when used, for example, with a
codon based mutagenesis method, which can be used to
produce random peptides or randomly or desirably biased
peptides (Huse, U.S. Patent No. 5,264,563, *supra*, 1993).
These or other well known methods can be used to produce
30   a phage display library, which can be subjected to *in
vivo* panning in order to identify tumor homing molecules
useful in the homing pro-apoptotic conjugates of the
invention.

52

In addition to screening a phage display library, *in vivo* panning can be used to screen various other types of libraries, including, for example, an RNA or DNA library or a chemical library. If desired, a
5 tumor homing molecule can be tagged, which can facilitate recovery of the molecule from the tumor or identification of the molecule in the tumor. For example, where a library of organic molecules, each containing a shared tag, is screened, the tag can be a moiety such as biotin,
10 which can be linked directly to the molecule or can be linked to a support containing the molecules. Biotin provides a shared tag useful for recovering the molecule from a selected tumor sample using an avidin or streptavidin affinity matrix. In addition, a molecule or
15 a support containing a molecule can be linked to a hapten such as 4-ethoxy-methylene-2-phenyl-2-oxazoline-5-one (phOx), which can be bound by an anti-phOx antibody linked to a magnetic bead as a means to recover the molecule. Methods for purifying biotin or phOx labeled
20 conjugates are known in the art and the materials for performing these procedures are commercially available (e.g., Invitrogen, La Jolla CA; and Promega Corp., Madison WI). In the case where a phage library is screened, the phage can be recovered using methods as
25 disclosed in Example IV.

In vivo panning provides a method for directly identifying tumor homing molecules that can selectively home to a tumor. As used herein, the term "home" or "selectively home" means that a particular molecule binds
30 relatively specifically to a target molecule present in the tumor following administration to a subject. In general, a tumor homing molecule is characterized, in part, by exhibiting at least a two-fold (2x) greater

53

specific binding to a tumor as compared to a control organ or tissue.

It should be recognized that, in some cases, a molecule can localize nonspecifically to an organ or
5 tissue containing a tumor. For example, *in vivo* panning of a phage display library can result in high background in organs such as liver and spleen, which contain a marked component of the reticuloendothelial system (RES). Thus, where a tumor is present, for example, in the
10 liver, nonspecific binding of molecules due to uptake by the RES can make identifying a tumor homing molecule more difficult.

Selective homing of a tumor homing molecule can be distinguished from nonspecific binding, however, by
15 detecting differences in the abilities of different individual phage to home to a tumor. For example, selective homing can be identified by combining a putative tumor homing molecule such as a peptide expressed on a phage with a large excess of non-infective
20 phage or with about a five-fold excess of phage expressing unselected peptides, injecting the mixture into a subject and collecting a sample of the tumor. In the latter case, for example, provided the number of injected phage expressing tumor homing peptide is
25 sufficiently low so as to be nonsaturating for the target molecule, a determination that greater than about 20% of the phage in the tumor express the putative tumor homing molecule is demonstrative evidence that the peptide expressed by the phage is a specific tumor homing
30 molecule. In addition, nonspecific localization can be distinguished from selective homing by performing competition experiments using, for example, phage expressing a putative tumor homing peptide in combination

54

with an excess amount of the "free" peptide
(Example VII).

In addition, various methods can be used to
prevent nonspecific binding of a molecule to an organ
5   containing a component of the RES. For example, a
molecule that homes selectively to a tumor present in an
organ containing a component of the RES can be obtained
by first blocking the RES using, for example, polystyrene
latex particles or dextran sulfate (see Kalin et al.,
10 Nucl. Med. Biol. 20:171-174 (1993); Illum et al.,
J. Pharm. Sci. 75:16-22 (1986); Takeya et al., J. Gen.
Microbiol. 100:373-379 (1977) , each of which is
incorporated herein by reference), then administering the
library to the subject. For example, pre-administration
15 of dextran sulfate 500 or polystyrene microspheres prior
to administration of a test substance has been used to
block nonspecific uptake of the test substance by Kupffer
cells, which are the RES component of the liver (Illum et
al., *supra*, 1986). Similarly, nonspecific uptake of
20 agents by the RES has been blocked using carbon particles
or silica (Takeya et al., *supra*, 1977) or a gelatine
colloid (Kalin et al., *supra*, 1993). Thus, various
agents useful for blocking nonspecific uptake by the RES
are known and routinely used.

25   Nonspecific binding of phage to RES or to other
sites also can be prevented by coinjecting, for example,
mice with a specific phage display library together with
the same phage made noninfective (Smith et al., *supra*,
1990, 1993). In addition, a peptide that homes to tumor
30 in an organ containing an RES component can be identified
by preparing a phage display library using phage that
exhibit low background binding to the particular organ.
For example, Merrill et al. (Proc. Natl. Acad. Sci., USA

93:3188-3192 (1996), which is incorporated herein by
reference) selected lambda-type phage that are not taken
up by the RES and, as a result, remain in the circulation
for a prolonged period of time. A filamentous phage

5   variant, for example, can be selected using similar
methods.

            Selective homing of a tumor homing molecule can
be demonstrated by determining the specificity of a tumor
homing molecule for the tumor as compared to a control

10 organ or tissue. Selective homing also can be
demonstrated by showing that molecules that home to a
tumor, as identified by one round of *in vivo* panning, are
enriched for tumor homing molecules in a subsequent round
of *in vivo* panning. For example, phage expressing

15 peptides that selectively home to a melanoma tumor were
isolated by *in vivo* panning, then were subjected to
additional rounds of *in vivo* panning. Following a second
round of screening, phage recovered from the tumor showed
a 3-fold enrichment in homing to the tumor as compared to

20 brain. Phage recovered from the tumor after a third
round of screening showed an average of 10-fold
enrichment in homing to the tumor as compared to brain.
Selective homing also can be demonstrated by showing that
molecules that home to a selected tumor, as identified by

25 one round of *in vivo* panning, are enriched for tumor
homing molecules in a subsequent round of *in vivo*
panning.

            Tumor homing molecules can be identified by *in
vivo* panning using, for example, a mouse containing a

30 transplanted tumor. Such a transplanted tumor can be,
for example, a human tumor that is transplanted into
immunodeficient mice such as nude mice or a murine tumor
that is maintained by passage in tissue culture or in

56

mice. Due to the conserved nature of cellular receptors
and of ligands that bind a particular receptor, it is
expected that angiogenic vasculature and histologically
similar tumor cells in various species can share common
5   cell surface markers useful as target molecules for a
tumor homing molecule. Thus, the skilled artisan would
recognize that a tumor homing molecule identified using,
for example, *in vivo* panning in a mouse having a murine
tumor of a defined histological type such as a melanoma
10 also would bind to the corresponding target molecule in a
tumor in a human or other species. Similarly, tumors
growing in experimental animals require associated
neovascularization, just as that required for a tumor
growing in a human or other species. Thus, a tumor
15 homing molecule that binds a target molecule present in
the vasculature in a tumor grown in a mouse likely also
can bind to the corresponding target molecule in the
vasculature of a tumor in a human or other mammalian
subject. The general ability of a tumor homing molecule
20 identified, for example, by homing to a human breast
tumor, also to home to a human Kaposi's sarcoma or to a
mouse melanoma indicates that the target molecules are
shared by many tumors. Indeed, the results disclosed
herein demonstrate that the target molecules are
25 expressed in the neovasculature, which is host tissue
(see Example VII).

        A tumor homing molecule identified using *in
vivo* panning in an experimental animal such as a mouse
readily can be examined for the ability to bind to a
30 corresponding tumor in a human patient by demonstrating,
for example, that the molecule also can bind specifically
to a sample of the tumor obtained from the patient. For
example, the CDCRGDCFC (SEQ ID NO: 1) phage and NGR
peptides have been shown to bind to blood vessels in

microscopic sections of human tumors, whereas little or
no binding occurs in the blood vessels of nontumor
tissues. Thus, routine methods can be used to confirm
that a tumor homing molecule identified using *in vivo*
5 panning in an experimental animal also can bind the
target molecule in a human tumor.

        The steps of administering the library to the
subject, collecting a selected tumor and identifying
tumor homing molecules that home to the tumor, comprise a
10 single round of *in vivo* panning. Although not required,
one or more additional rounds of *in vivo* panning
generally are performed. Where an additional round of *in
vivo* panning is performed, the molecules recovered from
the tumor in the previous round are administered to a
15 subject, which can be the same subject used in the
previous round, where only a part of the tumor was
collected.

        By performing a second round of *in vivo*
panning, the relative binding selectivity of the
20 molecules recovered from the first round can be
determined by administering the identified molecules to a
subject, collecting the tumor, and determining whether
more phage are recovered from the tumor following the
second round of screening as compared to those recovered
25 following the first round. Although not required, a
control organ or tissue also can be collected and the
molecules recovered from the tumor can be compared with
those recovered from the control organ. Ideally, no
molecules are recovered from a control organ or tissue
30 following a second or subsequent round of *in vivo*
panning. Generally, however, a proportion of the
molecules also will be present in a control organ or
tissue. In this case, the ratio of molecules in the

58

selected tumor as compared to the control organ
(selected:control) can be determined. For example, phage
that homed to melanoma following a first round of *in vivo*
panning demonstrated a 3x enrichment in homing to the
5   selected tumor as compared to the control organ, brain,
following two additional rounds of panning
(Example VIII).

        Additional rounds of *in vivo* panning can be
used to determine whether a particular molecule homes
10 only to the selected tumor or can recognize a target on
the tumor that also is expressed in one or more normal
organs or tissues in a subject or is sufficiently similar
to the target molecule on the tumor. It is unlikely that
a tumor homing molecule also will home to a corresponding
15 normal tissue because the method of *in vivo* panning
selects only those molecules that home to the selected
tumor. Where a tumor homing molecule also directs homing
to one or more normal organs or tissues in addition to
the tumor, the organs or tissues are considered to
20 constitute a family of selected organs or tissues. Using
the method of *in vivo* panning, molecules that home to
only the selected tumor can be distinguished from
molecules that also home to one or more selected organs
or tissues. Such identification is expedited by
25 collecting various organs or tissues during subsequent
rounds of *in vivo* panning.

        The term "control organ or tissue" is used to
mean an organ or tissue other than the tumor for which
the identification of a tumor homing molecule is desired.
30 A control organ or tissue is characterized in that a
tumor homing molecule does not selectively home to the
control organ. A control organ or tissue can be
collected, for example, to identify nonspecific binding

of the molecule or to determine the selectivity of homing
of the molecule. In addition, nonspecific binding can be
identified by administering, for example, a control
molecule, which is known not to home to a tumor but is
5 chemically similar to a potential tumor homing molecule.
Alternatively, where the administered molecules are
linked to a support, administration of the supports,
alone, also can be used to identify nonspecific binding.
For example, a phage that expresses the gene III protein,
10 alone, but that does not contain a peptide fusion
protein, can be studied by *in vivo* panning to determine
the level of nonspecific binding of the phage support.

        As disclosed herein, specific homing of a tumor
homing molecule readily can be identified by examining
15 the selected tumor tissue as compared to a corresponding
nontumor tissue, as well as to control organs or tissues.
For example, immunohistological analysis can be performed
on a tumor tissue and corresponding nontumor tissue using
an antibody specific for a phage used to display tumor
20 homing peptides (see Example VII). Alternatively, an
antibody can be used that is specific for a shared tag
that is expressed with the peptide, for example, a FLAG
epitope or the like, such detection systems being
commercially available.

25   In general, a library of molecules, which
contains a diverse population of random or selectively
randomized molecules of interest, is prepared, then
administered to a subject. At a selected time after
administration, the subject is sacrificed and the tumor
30 is collected such that the molecules present in the tumor
can be identified (see Example IV). If desired, one or
more control organs or tissues or a part of a control
organ or tissue can be sampled. For example, mice

60

bearing a breast tumor or a melanoma tumor were injected
with a phage peptide display library, then, after about
1 to 5 minutes, the mice were anesthetized, either frozen
in liquid nitrogen or, preferably, are perfused through
5 the heart to terminate circulation of the phage, the
tumor and one or more control organs were collected from
each, phage present in the tumor and the control organs
were recovered and peptides that selectively homed to the
respective tumors were identified (see Examples IV, V
10 and VIII).

        In the examples provided, the animals were
sacrificed to collect the selected tumor and control
organ or tissue. It should be recognized, however, that
only a part of a tumor need be collected to recover a
15 support containing a tumor homing molecule and,
similarly, only part of an organ or tissue need be
collected as a control. · Thus, a part of a tumor, for
example, can be collected by biopsy, such that a molecule
such as a peptide expressed by a phage can be
20 administered to the same subject a second time or more,
as desired. Where the molecule that is to be
administered a second time to the same subject is tagged
or linked, for example, to a support, the tag or support
should be nontoxic and biodegradable, so as not to
25 interfere with subsequent rounds of screening.

        In vitro screening of phage libraries
previously has been used to identify peptides that bind
to antibodies or to cell surface receptors (Smith and
Scott, *supra*, 1993). For example, *in vitro* screening of
30 phage peptide display libraries has been used to identify
novel peptides that specifically bound to integrin
adhesion receptors (Koivunen et al., <u>J. Cell Biol.</u>
124:373-380 (1994a), which is incorporated herein by

61

reference) and to the human urokinase receptor (Goodson
et al., _Proc. Natl. Acad. Sci., USA_ 91:7129-7133 (1994)).
However, such _in vitro_ studies provide no insight as to
whether a peptide that can specifically bind to a
5   selected receptor _in vitro_ also will bind the receptor _in
vivo_ or whether the binding peptide or the receptor are
unique to a specific organ in the body. Furthermore, the
in vitro methods are performed using defined,
well-characterized target molecules in an artificial
10 system. For example, Goodson et al., _supra_, 1994,
utilized cells expressing a recombinant urokinase
receptor. However, such _in vitro_ methods are limited in
that they require prior knowledge of the target molecule
and yield little if any information regarding _in vivo_
15 utility.

        In vitro panning against cells in culture also
has been used to identify molecules that can specifically
bind to a receptor expressed by the cells (Barry et al.,
Nature Med. 2:299-305 (1996), which is incorporated
20 herein by reference). However, the cell surface
molecules that are expressed by a cell _in vivo_ often
change when the cell is grown in culture. Thus, _in vitro_
panning methods using cells in culture also are limited
in that there is no guarantee a molecule that is
25 identified due to its binding to a cell in culture will
have the same binding ability _in vivo_. Furthermore, it
is not possible to use _in vitro_ panning to distinguish
molecules that home only to the tumor cells used in the
screening, but not to other cell types.

62

In contrast, *in vivo* panning requires no prior knowledge or availability of a target molecule and identifies molecules that bind to cell surface target molecules that are expressed *in vivo*. Also, since the
5 "nontargeted" tissues are present during the screening, the probability of isolating tumor homing molecules that lack specificity of homing is greatly reduced. Furthermore, in obtaining tumor homing molecules by *in vivo* panning, any molecules that may be particularly
10 susceptible to degradation in the circulation *in vivo* due, for example, to a metabolic activity, are not recovered. Thus, *in vivo* panning provides significant advantages over previous methods by identifying tumor homing molecules that selectively home *in vivo* to a
15 target molecule present in a tumor.

Although mechanisms by which the disclosed method of *in vivo* panning works have not been fully defined, one possibility is that a molecule such as a peptide expressed on a phage recognizes and binds to a
20 target molecule present on endothelial cells lining the blood vessels in a tumor. Evidence indicates, for example, that the vascular tissues in various organs differ from one another and that such differences can be involved in regulating cellular trafficking in the body.
25 For example, lymphocytes home to lymph nodes or other lymphoid tissues due, in part, to the expression of specific "address" molecules by the endothelial cells in those tissues (Salmi et al., Proc. Natl. Acad. Sci., USA 89:11436-11440 (1992); Springer, Cell 76:301-314 (1994)).
30 Similarly, various leukocytes can recognize sites of inflammation due, in part, to the expression of endothelial cell markers induced by inflammatory signals (see Butcher and Picker, Science 272:60-66 (1996);

Springer, *supra*, 1994). Thus, endothelial cell markers
provide a potential target that can be selectively bound
by a tumor homing molecule and used to direct a linked
antimicrobial peptide to a tumor.

5   Additional components can be included as part
of the homing pro-apoptotic conjugate, if desired. For
example, in some cases, it can be desirable to utilize an
oligopeptide spacer between a tumor homing molecule and
the antimicrobial peptide. Such spacers are well known
10 in the art, as described, for example, in Fitzpatrick and
Garnett, <u>Anticancer Drug Des.</u> 10:1-9 (1995)).

           A homing pro-apoptotic chimeric peptide of the
invention can readily be synthesized in required
quantities using routine methods of solid state peptide
15 synthesis. A chimeric peptide of the invention also can
be purchased from a commercial source (for example,
AnaSpec, Inc.; San Jose, CA). Where an antimicrobial
peptide is to be linked to a non-peptide tumor homing
molecule, the antimicrobial peptide portion can be
20 synthesized independently using well known methods or
obtained from commercial sources.

           Several methods can be used to link an
antimicrobial peptide to a tumor homing molecule are
known in the art, depending on the particular chemical
25 characteristics of the molecule. For example, methods of
linking haptens to carrier proteins as used routinely in
the field of applied immunology (see, for example, Harlow
and Lane, *supra*, 1988; Hermanson, *supra*, 1996).

64

A premade antimicrobial peptide also can be
conjugated to a tumor homing peptide using, for example,
carbodiimide conjugation (Bauminger and Wilchek, Meth.
Enzymol. 70:151-159 (1980), which is incorporated herein
5 by reference). Carbodiimides comprise a group of
compounds that have the general formula R-N=C=N-R', where
R and R' can be aliphatic or aromatic, and are used for
synthesis of peptide bonds. The preparative procedure is
simple, relatively fast, and is carried out under mild
10 conditions. Carbodiimide compounds attack carboxylic
groups to change them into reactive sites for free amino
groups. Carbodiimide conjugation has been used to
conjugate a variety of compounds to carriers for the
production of antibodies.

15   The water soluble carbodiimide,
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) can
be useful for conjugating an antimicrobial peptide to a
tumor homing molecule. Such conjugation requires the
presence of an amino group, which can be provided, for
20 example, by an antimicrobial peptide, and a carboxyl
group, which can be provided by the tumor homing
molecule.

In addition to using carbodiimides for the
direct formation of peptide bonds, EDC also can be used
25 to prepare active esters such as N-hydroxysuccinimide
(NHS) ester. The NHS ester, which binds only to amino
groups, then can be used to induce the formation of an
amide bond with the single amino group of the
doxorubicin. The use of EDC and NHS in combination is
30 commonly used for conjugation in order to increase yield
of conjugate formation (Bauminger and Wilchek, *supra*,
1980).

65

Other methods for conjugating an antimicrobial peptide to a tumor homing molecule also can be used. For example, sodium periodate oxidation followed by reductive alkylation of appropriate reactants can be used, as can
5 glutaraldehyde crosslinking. However, it is recognized that, regardless of which method of producing a conjugate of the invention is selected, a determination must be made that the tumor homing molecule maintains its targeting ability and that the antimicrobial peptide
10 maintains its antimicrobial activity. Methods known in the art can confirm the activity of a homing pro-apoptotic conjugate of the invention.

The yield of antimicrobial peptide/tumor homing molecule conjugate formed is determined using routine
15 methods. For example, HPLC or capillary electrophoresis or other qualitative or quantitative method can be used (see, for example, Liu et al., _J. Chromatogr._ 735:357-366 (1996); Rose et al., _J. Chromatogr._ 425:419-412 (1988), each of which is incorporated herein by reference; see,
20 also, Example VIII). In particular, the skilled artisan will recognize that the choice of a method for determining yield of a conjugation reaction depends, in part, on the physical and chemical characteristics of the specific antimicrobial peptide and tumor homing molecule.
25 Following conjugation, the reaction products are desalted to remove any free peptide or molecule.

The present invention also provides methods of directing an antimicrobial peptide _in vivo_ to a tumor having angiogenic vasculature. The method is practiced
30 by administering a homing pro-apoptotic conjugate of the invention to a subject containing a tumor having angiogenic vasculature. In a method of the invention for directing an antimicrobial peptide _in vivo_ to a tumor

66

having angiogenic vasculature, the antimicrobial peptide
can include, for example, the sequence $_D$(KLAKLAK)$_2$.
Particularly useful conjugates that can be administered
to a subject containing a tumor having angiogenic
5   vasculature include CNGRC-GG-$_D$(KLAKLAK)$_2$ and
ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$.

         The present invention additionally provides
methods of inducing selective toxicity *in vivo* in a tumor
having angiogenic vasculature. The methods are practiced
10 by administering a homing pro-apoptotic conjugate of the
invention to a subject containing a tumor having
angiogenic vasculature. An antimicrobial peptide useful
in inducing selective toxicity in a method of the
invention can be, for example, a peptide containing the
15 sequence $_D$(KLAKLAK)$_2$. Particularly useful conjugates that
can be administered to induce selective toxicity *in vivo*
in a tumor having angiogenic vasculature include
CNGRC-GG-$_D$(KLAKLAK)$_2$ and ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$.

         Also provided herein are methods of treating a
20 patient with a tumor having angiogenic vasculature. In
such methods of treatment, a homing pro-apoptotic
conjugate of the invention is administered to the patient
and is selectively toxic to the tumor. The antimicrobial
peptide portion can include, for example, the sequence
25 $_D$(KLAKLAK)$_2$. In preferred embodiments, the homing
pro-apoptotic conjugate has the sequence
CNGRC-GG-$_D$(KLAKLAK)$_2$ or ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$.

         When administered to a subject, a homing
pro-apoptotic conjugate of the invention can be
30 administered as a pharmaceutical composition containing,
for example, the conjugate and a pharmaceutically
acceptable carrier. Pharmaceutically acceptable carriers

are well known in the art and include, for example,
aqueous solutions such as water or physiologically
buffered saline or other solvents or vehicles such as
glycols, glycerol, oils such as olive oil or injectable
5 organic esters.

A pharmaceutically acceptable carrier can
contain physiologically acceptable compounds that act,
for example, to stabilize or to increase the absorption
of the conjugate. Such physiologically acceptable
10 compounds include, for example, carbohydrates, such as
glucose, sucrose or dextrans; antioxidants, such as
ascorbic acid or glutathione; chelating agents; low
molecular weight proteins; or other stabilizers or
excipients. One skilled in the art would know that the
15 choice of a pharmaceutically acceptable carrier,
including a physiologically acceptable compound, depends,
for example, on the route of administration of the
composition. The pharmaceutical composition also can
contain an agent such as a cancer therapeutic agent.

20 One skilled in the art would know that a homing
pro-apoptotic conjugate of the invention can be
administered as a pharmaceutical composition to a subject
by various routes including, for example, orally or
parenterally, such as intravenously. A pharmaceutical
25 composition containing the conjugate can be administered
by injection or by intubation. The pharmaceutical
composition also can be a tumor homing molecule linked to
liposomes or other polymer matrices, which can have
incorporated therein, an antimicrobial peptide
30 (Gregoriadis, Liposome Technology, Vol. 1 (CRC Press,
Boca Raton, FL 1984), which is incorporated herein by
reference). Liposomes, for example, which consist of
phospholipids or other lipids, are nontoxic,

68

physiologically acceptable and metabolizable carriers
that are relatively simple to make and administer.

        For the therapeutic methods disclosed herein,
an effective amount of the homing pro-apoptotic conjugate
5   must be administered to the subject. As used herein, the
term "effective amount" means the amount of the conjugate
that produces the desired effect. An effective amount
often will depend on the particular antimicrobial peptide
linked to the tumor homing molecule. An effective amount
10 of a homing pro-apoptotic conjugate in which a tumor
homing molecule is linked to a particular antimicrobial
peptide can be determined using methods well known to
those in the art.

        The route of administration of a homing
15 pro-apoptotic conjugate will depend, in part, on the
chemical structure of the molecule. Peptides, for
example, are not particularly useful when administered
orally because they can be degraded in the digestive
tract. However, methods for chemically modifying
20 peptides to render them less susceptible to degradation
by endogenous proteases or more absorbable through the
alimentary tract, including incorporation of D-amino
acids, are well known (see, for example, Blondelle et
al., *supra*, 1995; Ecker and Crooke, *supra*, 1995; Goodman
25 and Ro, *supra*, 1995). Such modifications can be
performed on tumor homing peptides identified by *in vivo*
panning as well as on antimicrobial peptides. In
addition, methods for preparing libraries of
peptidomimetics, which can contain D-amino acids, other
30 non-naturally occurring amino acids, or chemically
modified amino acids; or can be organic molecules that
mimic the structure of a peptide; or can be peptoids such
as vinylogous peptoids, are known in the art and can be

used to identify tumor homing molecules that are stable
for oral administration.

          A tumor homing peptide can have a linear or
cyclic structure. Cysteine residues were included in
5 some peptides, allowing cyclization of the peptides. In
particular, peptides containing at least two cysteine
residues cyclize spontaneously. In addition, such cyclic
peptides also can be active when present in a linear form
(see, for example, Koivunen et al., *supra*, 1993). For
10 example, the linear peptide, NGRAHA (SEQ ID NO: 6), also
was useful as tumor homing molecule (see Table 2). Thus,
in some cases one or more cysteine residues in the tumor
homing peptides disclosed herein or otherwise identified
as tumor homing peptides can be deleted without
15 significantly affecting the tumor homing activity of the
peptide. Methods for determining the necessity of a
cysteine residue or of amino acid residues N-terminal or
C-terminal to a cysteine residue for tumor homing
activity of a peptide of the invention are routine and
20 well known in the art.

          As further disclosed herein, some, but not all,
tumor homing molecules also can home to angiogenic
vasculature that is not contained within a tumor. For
example, tumor homing molecules containing either the RGD
25 motif or the GSL motif specifically homed to retinal
neovasculature (Smith et al., Invest. Ophthamol. Vis.
Sci. 35:101-111 (1994), which is incorporated herein by
reference), whereas tumor homing peptides containing the
NGR motif did not accumulate substantially in this
30 angiogenic vasculature. These results indicate that
tumor vasculature expresses target molecules that are not
substantially expressed by other kinds of angiogenic
vasculature. Methods as disclosed herein can be used to

70

distinguish tumor homing peptides from peptides that home
to nontumor angiogenic vasculature. One skilled in the
art understands that, preferably, for treatment of a
tumor, one administers a conjugate having a tumor homing
5 peptide, which selectively homes to tumor vasculature.

        The invention provides chimeric prostate-homing
pro-apoptotic peptides which can be used to treat, for
example, benign prostate hyperplasia or cancer of the
prostate. As disclosed herein, SMSIARL peptide (SEQ ID
10 NO: 207) can selectively localize to prostate tissue,
specifically prostate vasculature, when systemically
administered (see Example IX.B and IX.E). Furthermore,
the prostate homing peptide SMSIARL (SEQ ID NO: 207) can
be used to selectively deliver a linked moiety, such as
15 biotin or phage, to prostate tissue. As further
disclosed herein, apoptosis was induced in mouse prostate
by systemic administration of SMSIARL-GG-$_D$(KLAKLAK)$_2$
chimeric peptide; no evidence of apoptosis was observed
in non-prostate tissues (see Figure 7 and Example IX.C).
20 The results disclosed herein also demonstrate that
administration of SMSIARL-GG-$_D$(KLAKLAK)$_2$ chimeric peptide
can extend the survival of TRAMP mice, which develop
prostate cancer under the influence of a transgene as
described in Gingrich et al., Cancer Res. 56: 4096-4102
25 (1996). Figure 8 shows that
SMSIARL-GG-$_D$(KLAKLAK)$_2$-treated mice survived longer than
mice treated with vehicle alone, $_D$(KLAKLAK)$_2$ peptide
alone, or SMSIARL peptide (SEQ ID NO: 207) alone. Based
on these results, the invention provides a chimeric
30 prostate-homing pro-apoptotic peptide as well as methods
of using the peptide to treat a patient having prostate
cancer as described further below.

71

   Thus, the present invention provides a chimeric
prostate-homing pro-apoptotic peptide that contains a
prostate-homing peptide linked to an antimicrobial
peptide, where the chimeric peptide is selectively
5 internalized by prostate tissue and exhibits high
toxicity thereto, while the antimicrobial peptide has low
mammalian cell toxicity when not linked to the
prostate-homing peptide. In a chimeric peptide of the
invention, the prostate-homing peptide portion can
10 contain, for example, the sequence SMSIARL (SEQ ID NO:
207) or a functionally equivalent sequence, and the
antimicrobial peptide portion can have an amphipathic
α-helical structure such as the sequence $(KLAKLAK)_2$ (SEQ
ID NO: 200), $(KLAKKLA)_2$ (SEQ ID NO: 201), $(KAAKKAA)_2$ (SEQ
15 ID NO: 202) or $(KLGKKLG)_3$ (SEQ ID NO: 203). In a
preferred embodiment, the antimicrobial peptide portion
contains the sequence $_D(KLAKLAK)_2$. An exemplary
prostate-homing pro-apoptotic peptide is provided herein
as $SMSIARL\text{-}GG\text{-}_D(KLAKLAK)_2$.

20   The present invention further provides a method
of directing an antimicrobial peptide in vivo to a
prostate cancer. The method includes the step of
administering a chimeric prostate-homing pro-apoptotic
peptide that contains a prostate-homing peptide linked to
25 an antimicrobial peptide, where the chimeric peptide is
selectively internalized by prostate tissue and exhibits
high toxicity thereto, while the antimicrobial peptide
has low mammalian cell toxicity when not linked to the
prostate-homing peptide. In a method of the invention,
30 the prostate-homing peptide can contain, for example, the
sequence SMSIARL (SEQ ID NO: 207) or a functionally
equivalent sequence, and the antimicrobial peptide can
contain a sequence such as $_D(KLAKLAK)_2$. In a preferred

72

embodiment, the chimeric prostate-homing pro-apoptotic
peptide includes the sequence SMSIARL-GG-$_D$(KLAKLAK)$_2$.

      Also provided by the invention is a method of
inducing selective toxicity *in vivo* in a prostate cancer.
5   The method includes the step of administering to a
subject containing a prostate cancer a chimeric
prostate-homing pro-apoptotic peptide that contains a
prostate-homing peptide linked to an antimicrobial
peptide, where the chimeric peptide is selectively
10 internalized by prostate tissue and exhibits high
toxicity thereto, while the antimicrobial peptide has low
mammalian cell toxicity when not linked to the
prostate-homing peptide. The method of inducing
selective toxicity *in vivo* in a prostate cancer can be
15 practiced, for example, with a prostate-homing peptide
containing the sequence SMSIARL (SEQ ID NO: 207) or a
functionally equivalent sequence. The antimicrobial
peptide can include, for example, the sequence
$_D$(KLAKLAK)$_2$. In a preferred embodiment, the chimeric
20 prostate-homing pro-apoptotic peptide includes the
sequence SMSIARL-GG-$_D$(KLAKLAK)$_2$.

      In addition, the invention provides a method of
treating a patient having prostate cancer by
administering to the patient a chimeric prostate-homing
25 pro-apoptotic peptide of the invention, whereby the
chimeric peptide is selectively toxic to the tumor. The
chimeric peptide contains a prostate-homing peptide
linked to an antimicrobial peptide, and the chimeric
peptide is selectively internalized by prostate tissue
30 and exhibits high toxicity thereto, while the
antimicrobial peptide has low mammalian cell toxicity
when not linked to the prostate-homing peptide. The
prostate-homing peptide portion can contain, for example,

the sequence SMSIARL (SEQ ID NO: 207) or a functionally
equivalent sequence, and the antimicrobial peptide
portion can contain, for example, the sequence
$_D$(KLAKLAK)$_2$. In a preferred embodiment for treating a
5 patient having a prostate tumor, the chimeric peptide
contains the sequence SMSIARL-GG-$_D$(KLAKLAK)$_2$.

        As used herein, the term "prostate-homing
peptide" means a peptide that selectively homes *in vivo*
to prostate tissue as compared to control tissue, such as
10 brain. Such a peptide generally is characterized by at
least a two-fold greater localization to prostatic tissue
as compared to a control cell type or tissue. A prostate
homing peptide can selectively home, for example, to
prostate vasculature as compared to other cell types or
15 other vasculature (see Example IX).

        A chimeric peptide of the invention is
selectively delivered to the prostate due to the
selective homing activity of the prostate-homing peptide
portion. A variety of prostate-homing peptides are
20 useful in the invention, including SMSIARL (SEQ ID
NO: 207) and VSFLEYR (SEQ ID NO: 222), which were
identified by injection of an X$_7$ library into mice (Table
7) and subsequent *in vivo* panning as described in U.S.
Patent No. 5,622,699. The prostate homing peptides
25 SMSIARL (SEQ ID NO: 21) and VSFLEYR (SEQ ID NO: 22)
exhibited a 34-fold and 17-fold enrichment, respectively,
in prostate as compared to brain.

        In one embodiment, the invention relies on a
prostate-homing peptide which contains the sequence
30 SMSIARL (SEQ ID NO: 207), or a functionally equivalent
sequence. The term "functionally equivalent sequence,"
as used herein in reference to the sequence SMSIARL (SEQ

ID NO: 207), means a sequence that binds selectively to
the endothelium of prostatic blood vessels, as shown in
Figure 9 for the sequence SMSIARL (SEQ ID NO: 207), and
that functions similarly in that the sequence binds
5 selectively to the same receptor.

It is understood that the chimeric
prostate-homing pro-apoptotic peptides of the invention
can be used to induce selective toxicity in a variety of
prostatic disorders. Such disorders include benign
10 nodular hyperplasia of the prostate as well as primary or
secondary cancers including clinically apparent as well
as subclinical cancers. Cancers to be treated with a
chimeric peptide of the invention include prostatic
carcinomas such as adenocarcinomas.

15   The following examples are intended to
illustrate but not limit the present invention.

EXAMPLE I
CHARACTERIZATION OF $_D$(KLAKLAK)$_2$

This example demonstrates that $_D$(KLAKLAK)$_2$
20 preferentially disrupts mitochondrial membrane and
induces mitochondria-dependent apoptosis.

The synthetic 14-mer KLAKLAKKLAKLAK (SEQ ID
NO: 200), designated (KLAKLAK)$_2$, was selected because it
25 kills bacteria at a concentration two orders of magnitude
lower than the concentration required to kill eukaryotic
cells (Javadpour et al., _J. Med. Chem._ 39:3107-3113
(1996)). The all D-enantiomer, $_D$(KLAKLAK)$_2$, was used to
avoid degradation by proteases (Bessalle et al.,
30 _FEBS Lett._ 274:151-155 (1990); Wade et al., _Proc. Natl._
Acad. Sci. 87:4761-4765 (1990)).

ᴅ(KLAKLAK)₂ preferentially disrupts mitochondrial
membranes

          The ability of ᴅ(KLAKLAK)₂ to disrupt
mitochondrial membranes preferentially over eukaryotic
5 plasma membranes was evaluated by mitochondrial swelling
assays and in a mitochondria-dependent cell-free system
of apoptosis, and by cytotoxicity assays.

          Mitochondrial swelling assays were performed as
follows. Briefly, rat liver mitochondria were prepared
10 as described in Ellerby et al., J. Neurosci. 17:6165-6178
(1997). Peptides were synthesized at higher than 90%
purity by HPLC (DLSLARLATARLAI (SEQ ID NO: 204), Coast
Scientific, Inc., San Diego, CA; all other peptides,
AnaSpec, Inc.). Mitochondria were treated with a
15 concentration of 10 µM ᴅ(KLAKLAK)₂, 10 µM DLSLARLATARLAI
negative control peptide (SEQ ID NO: 204), or 200 µM Ca^{+2}
as a positive control. Peptides were added to
mitochondria in a cuvette, and swelling was quantified by
measuring the optical absorbance at 520 nm.
20

          As shown in Figure 2a, 10 µM ᴅ(KLAKLAK)₂ induced
marked mitochondrial swelling. Mild swelling was evident
at a concentration of 3 µM, two orders of magnitude less
than the concentration required to kill eukaryotic cells
25 (approximately 300 µM), as measured by the lethal
concentration required to kill 50% of a cell monolayer
(LC_{50}; Table 1). The non-α-helix forming peptide
DLSLARLATARLAI (SEQ ID NO: 204) used as a negative
control did not induce mitochondrial swelling. These
30 results demonstrate that ᴅ(KLAKLAK)₂ preferentially
disrupts mitochondrial membranes as compared to
eukaryotic plasma membranes.

TABLE 1

LC$_{50}$ (µM) FOR EUKARYOTIC CELLS TREATED
WITH HPP-1 AND $_D$(KLAKLAK)$_2$

	DMEC			KS1767	MDA-MB-435
	Angiostatic	Angiogenic			
	Prolif.	Prolif.	Cord Form	Prolif.	Prolif.
HPP-1	481	51	34	42	415
$_D$(KLAKLAK)$_2$	492	346	368	387	333

Results are means of three independent experiments performed in
triplicate (t-test, $P<0.03$).

_$_D$(KLAKLAK)$_2$ induces mitochondrial-dependent apoptosis_

        The $_D$(KLAKLAK)$_2$ peptide was assayed for the
ability to activate mitochondria-dependent apoptosis in a
cell-free system composed of normal mitochondria
suspended in normal cytosolic extract (Ellerby et al.,
J. Neurosci. 17:6165-6178 (1997)). Apoptosis was
measured by characteristic caspase-3 processing from an
inactive proform to the active protease (Alnemri et al.,
Cell 87:171 (1996)).

        The cell-free apoptosis assays were performed
essentially as follows. The cell-free systems were
reconstituted as described in Ellerby et al., *supra,*
1997, and, for mitochondria-dependent reactions, rat
liver mitochondria were suspended in normal
(non-apoptotic) cytosolic extract prepared from dermal
microvessel endothelial cells. After adding peptides at
a concentration of 100 µM and incubating for 2 hours at
30°C or 37°C, mitochondria were removed by centrifugation,
and the supernatant analyzed by SDS/PAGE immunoblotting
on a 12% gel (Biorad; Hercules, CA). Proteins were

transferred to PVDF membranes (Biorad) and incubated with
anti-caspase-3 antibody (Santa Cruz Biotechnology; Santa
Cruz, CA), followed by ECL detection (Amersham; Arlington
Heights, IL).

5   Characteristic caspase-3 processing was
measured in dermal microvessel endothelial cell lysates
as described in Ellerby et al., *supra,* 1997. Briefly,
aliquots of cell lysates (1 µl lysate, 8-15 mg/ml) were
added to 100 µM N-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA;
10   BioMol; 100 µl, 100 mM HEPES, 10% sucrose, 0.1% CHAPS,
1 mM DTT, pH 7.0). Hydrolysis of DEVD-pNA was monitored
spectrophotometrically (400 nm) at 25°C.

           As shown in Figure 2b, lane 4, characteristic
caspase-3 processing the active protease was observed in
15   the presence of mitochondria and $_D$(KLAKLAK)$_2$. The
non-α-helix forming peptide DLSLARLATARLAI (SEQ ID NO:
204) used as a negative control was inactive when tested
in the cell-free system and was not lethal to eukaryotic
cells (Figure 2b; see, also, Ellerby et al., *supra,*
20   1997).

           In sum, these results indicate that $_D$(KLAKLAK)$_2$
preferentially disrupts mitochondrial membranes as
compared to eukaryotic plasma membranes and activates
mitochondria-dependent apoptosis.

25   **EXAMPLE II**

                  <u>CHARACTERIZATION OF HPP-1</u>

           This example demonstrates that
CNGRC-GG-$_D$(KLAKLAK)$_2$ (HPP-1) inhibits angiogenesis in a
tissue culture model.

Chimeras were prepared containing a homing
domain linked through a glycinylglycine bridge to an
antimicrobial peptide. As described above, peptides were
synthesized commercially to higher than 90% purity by
5 HPLC by AnaSpec, Inc.

The homing domain was either the cyclic
(disulfide bond between cysteines) CNGRC peptide (SEQ ID
NO: 8; see Figure 1), or the double cyclic ACDCRGDCFC
peptide (SEQ ID NO: 16), both of which have tumor-homing
10 properties (Pasqualini et al., Nature Biotech. 15:542-546
(1997); Arap et al., Science 279:377-380 (1998)) and can
be internalized (Arap et al., *supra,* 1998; Hart et al.,
J. Biol. Chem. 269:12468-12474 (1994); Bretscher et al.,
EMBO . J. 8:1341-1348 (1989)). Due to the presumed
15 chiral nature of the homing domain-receptor interaction,
the homing domain was synthesized from all L amino acids.
The glycinylglycine bridge was used to couple the homing
and antimicrobial domains to impart peptide flexibility
and minimize potential steric interactions.

20 *Viability of dermal microvessel endothelial cells treated*
    with HPP-1

Efficacy and specificity of HPP-1 were
evaluated in a tissue culture model of angiogenesis as
described in Goto et al., Lab. Invest. 69:508-517 (1993).
25 During angiogenesis, capillary endothelial cells
proliferate and migrate (Risau, Nature 386:671-674
(1997); Zetter, Ann. Rev. Med. 49:407-424 (1998)). Cord
formation is a form of migration that is represented *in*
vitro by a change in endothelial cell morphology from the
30 usual "cobblestones" to chains or cords of cells as shown
in Figure 3 (see, also, Goto et al., *supra,* 1993).

The effect of HPP-1 was assayed on normal human
dermal microvessel endothelial cells (DMECs) under the
angiogenic conditions of proliferation and cord
formation. In addition, the effect of HPP-1 was assayed
5 under the angiostatic condition of a monolayer maintained
at 100% confluency (as described below).

Briefly, dermal microvessel endothelial cells
(DMECs) were grown in CADMEC Growth Media™(Cell
Applications, Inc.; San Diego, CA). Dermal microvessel
10 endothelial cells were then cultured under three
experimental conditions: proliferation, 30% confluency in
a growth media supplemented with 500 ng/ml human
recombinant vascular endothelial growth factor (VEGF;
Pharmingen); no proliferation, 100% confluency in media
15 formulated to maintain a monolayer; and cord formation,
60% confluency (required for induction) in media
formulated to induce cord formation. The KS1767 and
MDA-MB-435 cells were cultured as described in Arap et
al., *supra*, 1998; Hernier et al., *supra*, 1994).

20   Percent viability and LC_{50} (Table 1) were
determined by apoptotic morphology as described in
Ellerby et al., <u>J. Neurosci.</u> 17:6165-6178 (1997). For
the percent viability assays, dermal microvessel
endothelial cells were treated with 60 µM HPP-1 or
25 control peptide $_D$(KLAKLAK)$_2$. At the indicated time
points, cell culture medium was aspirated from adherent
cells, and the cells were gently washed once with PBS at
37°C. Subsequently, a 20-fold dilution of the dye
mixture (100 µg/ml acridine orange and 100 µg/ml ethidium
30 bromide) in PBS was gently pipetted on the cells, which
were viewed on an inverted microscope (Nikon TE 300).
Cells with nuclei exhibiting margination and condensation
of the chromatin and/or nuclear fragmentation (early/mid

80

apoptosis) or with compromised plasma membranes (late apoptosis) were scored as not viable. At least 500 cells were scored for a given time point in each experiment. Percent viability was calculated relative to untreated
5 controls. The LC_{50} for monolayer, proliferation (60% confluency), and cord formation were scored at 72 hours.

As shown in Figure 3, treatment of dermal microvessel endothelial cells with 60 mM HPP-1 led to a decrease in percent viability with time relative to
10 untreated controls, under the conditions of proliferation or cord formation (see Figures 3c and 3d, respectively). In contrast, treatment with the untargeted peptide $_D$(KLAKLAK)$_2$ as a negative control led to a negligible loss in viability. Furthermore, the LC_{50} for proliferating or
15 migrating dermal microvessel endothelial cells treated with HPP-1 was an order of magnitude lower than the LC_{50} for angiostatic dermal microvessel endothelial cells maintained in a monolayer at 100% confluency (Table 1). These results demonstrate preferential killing by HPP-1
20 under angiogenic conditions as compared to angiostatic conditions.

Various controls also were assayed for an effect on viability of dermal microvessel endothelial cells. The LC_{50} for the untargeted control $_D$(KLAKLAK)$_2$
25 under angiogenic conditions was similar to the LC_{50} for HPP-1 under angiostatic conditions. Furthermore, a mixture of uncoupled $_D$(KLAKLAK)$_2$ and CNGRC (SEQ ID NO: 8), a non-targeted form CARAC-GG-$_D$(KLAKLAK)$_2$, and a scrambled form CGRNC-GG-$_D$(KLAKLAK)$_2$ gave results similar to
30 $_D$(KLAKLAK)$_2$. Moreover, the alternative prototype ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ gave results similar to those for CNGRC-GG-$_D$(KLAKLAK)$_2$ (HPP-1; data not shown).

Mitochondrial morphology of dermal microvessel
endothelial cells treated with HPP-1

Mitochondrial morphology of dermal microvessel
endothelial cells was assessed in proliferating cells
5   after treatment with 60 µM HPP-1,
ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ or untargeted $_D$(KLAKLAK)$_2$ as
follows. Dermal microvessel endothelial cells were
stained after 24 and 72 hours treatment with peptide by
incubation for 30 min at 37°C with 100 nM mitochondrial
10 stain MitoTracker Red™ (Molecular Probes, Inc., Eugene ,
OR) and 500 nM of the nuclear stain DAPI (Molecular
Probes, Inc.). Mitochondria were subsequently visualized
using fluorescence microscopy (100x) under an inverted
microscope using a triple wavelength filter set (Nikon).

15   The mitochondria of dermal microvessel
endothelial cells treated for 24 hours with $_D$(KLAKLAK)$_2$
remained morphologically normal, while those treated with
each prototype exhibited altered mitochondrial
morphology. In particular, altered mitochondrial
20 morphology was evident in approximately 80% of cells
treated with CNGRC-GG-$_D$(KLAKLAK)$_2$ or
ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ before the cells rounded-up.
Ultimately, the CNGRC-GG-$_D$(KLAKLAK)$_2$ (HPP-1) treated
dermal microvessel endothelial cells exhibited the
25 classic morphological indicators of apoptosis, including
nuclear condensation and fragmentation, as seen at 72
hours (Ellerby et al., *supra,* 1997). Apoptotic cell
death was confirmed with by assaying for caspase activity
(see Figure 3b; Ellerby et al., *supra,* 1997).

82

Viability of KS1767 and MDA-MB-435 cells treated with
HPP-1

The chimeric HPP-1 peptide was as toxic to
5   KS1767 cells, which are derived from Kaposi's sarcoma, as
to the proliferating or migrating dermal microvessel
endothelial cells (Table 1; Hernier et al., AIDS 8:
575-581 (1994)). In contrast, HPP-1 was less toxic to
MDA-MB-435 human breast carcinoma cells by approximately
10   one order of magnitude (Table 1). KS1767 cells, which
are of endothelial origin, resemble angiogenic
endothelial cells and bind the CNGRC peptide (SEQ ID NO:
8), whereas MDA-MB-435 cells do not (Samaniego et al.,
Amer. J. Path. 152:1433-1443 (1998); Arap et al., *supra,*
15   1998).

In sum, these results demonstrate that HPP-1
induces mitochondrial swelling and apoptosis in dermal
microvessel endothelial cells.

20   **EXAMPLE III**
       IN VIVO ACTIVITY OF HOMING PRO-APOPTOTIC PEPTIDES

This example demonstrates that
CNGRC-GG-$_D$(KLAKLAK)$_2$ (HPP-1) inhibits tumor growth and
prolongs survival of tumor bearing animals. This example
25   further demonstrates that CDCRGDCFC-GG-$_D$(KLAKLAK)$_2$
inhibits retinal neovascularization.

83

A. *In vivo* activity of CNGRC-GG-$_D$(KLAKLAK)$_2$ (HPP-1)

          The activity of HPP-1 was tested *in vivo* using
nude mice bearing human MDA-MD-435 breast carcinoma
xenografts as follows. Briefly, MDA-MB-435 and
5 C8161-derived tumor xenografts were established in 2
month-old female nude mice (Jackson Labs; Bar Harbor, ME)
as described in Arap et al., *supra,* 1998. After mice
were anesthetized with a mixture of 2,2,2-tribromoethanol
(Aldrich; Milwaukee, WI) and 2-methyl-butanol (Aldrich)
10 prepared in distilled water, peptides were administered
intravenously through the tail vein at a dose of 250
µg/week/mouse given slowly in a volume of 200 µl.
Three-dimensional measurements of tumors were taken by
caliper under anesthesia and used to calculate tumor
15 volume (Pasqualini et al., *supra,* 1996).

          As shown in Figure 4a, tumor volume was
smaller, on average by one order of magnitude, than in
controls, which were a non-targeted CARAC-GG-$_D$(KLAKLAK)$_2$
and a mixture of uncoupled $_D$(KLAKLAK)$_2$ and CNGRC (SEQ ID
20 NO: 8) peptides. As further shown in Figure 4b, survival
was longer in the HPP-1 treated groups than in control
groups. Some of the HPP-1 treated mice outlived control
mice by several months, indicating that both primary
tumor growth and metastasis were inhibited by HPP-1.
25 Histopathological analysis revealed pronounced
destruction of tumor architecture and widespread cell
death in the tumors, demonstrating that the cell death
was approximately equally apoptotic and necrotic. In
similar experiments, HPP-1 was also effective against
30 tumors derived from the human melanoma cell line C8161
(Welch et al., Int. J. Cancer 47:227-237 (1991)).

84

These results indicate that homing
pro-apoptotic conjugates such as CNGRC-GG-$_D$(KLAKLAK)$_2$
(HPP-1) have strong anti-tumor activities *in vivo.*

B. *In vivo* activity of CDCRGDCFC-GG-$_D$(KLAKLAK)$_2$

5   Retinal angiogenesis was oxygen-induced in
newborn mice. Mice were subsequently treated with a
single 13 μg intravenous dose (one animal per group) of
vehicle; CDCRGDCFC-GG-$_D$(KLAKLAK)$_2$; or a control mixture of
unconjugated CDCRGDCFC (SEQ ID NO: 1) and $_D$(KLAKLAK)$_2$.
10 Four days later, retinal neovessel number was determined
for each treatment.

The results shown in Figure 5 demonstrated that
the number of retinal neovessels was reduced in mice
treated with the homing pro-apoptotic peptide
15 CDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ (column 2; striped bar) as
compared to mice treated with vehicle alone (column 1;
black bar) or with untargeted pro-apoptotic peptide
(column 3; hatched bar). In particular, the angiogenic
response in mice treated with the homing pro-apoptotic
20 peptide CDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ was only 30-40% of the
response seen in the control mice.

These results show that a homing pro-apoptotic
peptide such as CDCRGDCFC-GG-$_D$(KLAKLAK)$_2$ can selectively
inhibit an angiogenic response such as retinal
25 neovascularization.

85

EXAMPLE IV

IN VIVO PANNING

This example demonstrates methods for preparing
a phage library and screening the library using *in vivo*
5   panning to identify phage expressing tumor homing
peptides.

A. Preparation of phage libraries:

Phage display libraries were constructed using
the fuse 5 vector as described by Koivunen et al. (*supra*,
10 1995; Koivunen et al., *supra*, 1994b). Libraries encoding
peptides designated CX_5C (SEQ ID NO: 9), CX_6C (SEQ ID
NO: 10), CX_7C (SEQ ID NO: 11) and $CX_3CX_3CX_3C$ (SEQ ID
NO: 12) were prepared, where "C" indicates cysteine and
"X_N" indicates the given number of individually selected
15 amino acids. These libraries can display cyclic peptides
when at least two cysteine residues are present in the
peptide. In addition, a library that did not contain
defined cysteine residues also was constructed. Such a
library results in the production primarily of linear
20 peptides, although cyclic peptides also can occur due to
random probability.

A biased library based on the sequence
CXXXNGRXX (SEQ ID NO: 13) also was constructed.
Furthermore, in some cases the CXXXNGRXX (SEQ ID NO: 13)
25 library was further biased by in the incorporation of
cysteine residues flanking the NGR sequence, i.e.,
CXXCNGRCX (SEQ ID NO: 14; see Table 2).

86

The libraries containing the defined cysteine residues were generated using oligonucleotides constructed such that "C" was encoded by the codon TGT and "X_N" was encoded by NNK, where "N" is equal molar

5   mixtures of A, C, G and T, and where "K" is equal molar mixtures of G and T. Thus, the peptide represented by CX_5C (SEQ ID NO: 9) can be represented by an oligonucleotide having the sequence TGT(NNK)$_5$TGT (SEQ ID NO: 14). Oligonucleotides were made double stranded by 3

10 cycles of PCR amplification, purified and ligated to the nucleic acid encoding the gene III protein in the fuse 5 vector such that, upon expression, the peptide is present as a fusion protein at the N-terminus of the gene III protein.

15   The vectors were transfected by electroporation into MC1061 cells. Bacteria were cultured for 24 hr in the presence of 20 µg/ml tetracycline, then phage were collected from the supernatant by precipitation twice using polyethylene glycol. Each library contained about

20 5×10^9 to 5×10^{14} transducing units (TU; individual recombinant phage).

    B. In vivo panning of phage:

            Tumors were transplanted into mice as described in Examples V and VI, below. A mixture of phage

25 libraries containing 1×10^9 to 1×10^{14} TU was diluted in 200 µl DMEM and injected into the tail vein of anesthetized mice (AVERTIN (0.015 ml/g); see U.S. Patent No. 5,622,699; Pasqualini and Ruoslahti, *supra*, 1996). After 1-4 minutes, mice were snap frozen in liquid

30 nitrogen. To recover the phage, carcasses were partially thawed at room temperature for 1 hr, tumors and control organs were collected and weighed, then were ground in

1 ml DMEM-PI (DMEM containing protease inhibitors (PI);
phenylmethylsulfonyl fluoride (PMSF; 1 mM), aprotinin
(20 µg/ml), leupeptin (1 µg/ml)).

5   Alternatively, following introduction of a
library into a mouse, circulation of the library is
terminated by perfusion through the heart. Briefly, mice
were anesthetized with AVERTIN, then the heart was
exposed and a 0.4 mm needle connected through a 0.5 mm
cannula to a 10 cc syringe was inserted into the left
10 ventricle. An incision was made on the right atrium and
5 to 10 ml of DMEM was slowly administered, perfusing the
whole body over about a 5 to 10 min period. Efficiency
of the perfusion was monitored directly by histologic
analysis.

15   Tumor and organ samples were washed 3 times
with ice cold DMEM-PI containing 1% bovine serum albumin
(BSA), then directly incubated with 1 ml K91-kan bacteria
for 1 hr. Ten ml NZY medium containing 0.2 µg/ml
tetracycline (NZY/tet) was added to the bacterial
20 culture, the mixture was incubated in a 37°C shaker for
1 hr, then 10 µl or 100 µl aliquots were plated in agar
plates containing 12.5 µg/ml tetracycline (tet/agar).

        Individual colonies containing phage recovered
from a tumor were grown for 16 hr in 5 ml NZY/tet. The
25 bacterial cultures obtained from the individual colonies
were pooled and the phage were purified and re-injected
into mice as described above for a second round of *in
vivo* panning. In general, a third round of panning also
was performed. Phage DNA was purified from individual
30 bacterial colonies obtained from the final round of *in
vivo* panning and the DNA sequences encoding the peptides

88

expressed by selected phage were determined (see Koivunen et al., *supra*, 1994b).

EXAMPLE V
IDENTIFICATION OF TUMOR HOMING PEPTIDES
5   BY *IN VIVO* PANNING AGAINST A BREAST TUMOR

This example demonstrates that *in vivo* panning can be performed against a breast tumor to identify tumor homing peptides that home to various tumors.

Human 435 breast carcinoma cells (Price et al.,
10 *Cancer Res.* 50:717-721 (1990)) were inoculated into the mammary fat pad of nude mice. When the tumors attained a diameter of about 1 cm, either a phage targeting experiment was performed, in which phage expressing a specific peptide were administered to the tumor bearing
15 mouse, or *in vivo* panning was performed.

The breast tumor bearing mice were injected with 1 x 10^9 phage expressing a library of $CX_3CX_3CX_3C$ (SEQ ID NO: 12) peptides, where X_3 indicates three groups of independently selected, random amino acids. The phage
20 were allowed to circulate for 4 min, then the mice were anesthetized, snap frozen in liquid nitrogen while under anesthesia, and the tumor was removed. Phage were isolated from the tumor and subjected to two additional rounds of *in vivo* panning.

25

TABLE 2	

PEPTIDES FROM PHAGE RECOVERED FROM HUMAN BREAST CANCER

CGRECPRLCQSSC (2*)	CNGRCVSGCAGRC (3)	
CGEACGGQCALPC (20)	IWSGYGVYW (21)	
PSCAYMCIT (22)	WESLYFPRE (23)	
SKVLYYNWE (24)	CGLMCQGACFDVC (25)	
CERACRNLCREGC (26)	CPRGCLAVCVSQC (27)	
CKVCNGRCCG (28)	CEMCNGRCMG (29)	CPLCNGRCAL (30)
CPTCNGRCVR (31)	CGVCNGRCGL (32)	CEQCNGRCGQ (33)
CRNCNGRCEG (34)	CVLCNGRCWS (35)	CVTCNGRCRV (36)
CTECNGRCQL (37)	CRTCNGRCLE (38)	CETCNGRCVG (39)
CAVCNGRCGF (40)	CRDLNGRKVM (41)	CSCCNGRCGD (42)
CWGCNGRCRM (43)	CPLCNGRCAR (44)	CKSCNGRCLA (45)
CVPCNGRCHE (46)	CQSCNGRCVR (47)	CRTCNGRCQV (48)
CVQCNGRCAL (49)	CRCCNGRCSP (50)	CASNNGRVVL (51)
CGRCNGRCLL (52)	CWLCNGRCGR (53)	CSKCNGRCGH (54)
CVWCNGRCGL (55)	CIRCNGRCSV (56)	CGECNGRCVE (57)
CEGVNGRRLR (58)	CLSCNGRCPS (59)	CEVCNGRCAL (60)
CGSLVRC (5)	GRSQMQI (61)	HHTRFVS (62)
SKGLRHR (63)	VASVSVA (64)	WRVLAAF (65)
KMGPKVW (66)	IFSGSRE (67)	SPGSWTW (68)
NPRWFWD (69)	GRWYKWA (70)	IKARASP (71)
SGWCYRC (72)	ALVGLMR (73)	LWAEMTG (74)
CWSGVDC (75)	DTLRLRI (76)	SKSSGVS (77)
IVADYQR (78)	VWRTGHL (79)	VVDRFPD (80)
LSMFTRP (81)	GLPVKWS (82)	IMYPGWL (83)
CVMVRDGDC (84)	CVRIRPC (85)	CQLAAVC (86)
CGVGSSC (87)	CVSGPRC (88)	CGLSDSC (89)
CGEGHPC (90)	CYTADPC (91)	CELSLISKC (92)
CPEHRSLVC (93)	CLVVHEAAC (94)	CYVELHC (95)
CWRKFYC (96)	CFWPNRC (97)	CYSYFLAC (98)
CPRGSRC (99)	CRLGIAC (100)	CDDSWKC (101)
CAQLLQVSC (102)	CYPADPC (103)	CKALSQAC (104)
CTDYVRC (105)	CGETMRC (106)	

* - numbers in parentheses indicate SEQ ID NO:.

Following the third round of panning, phage
were quantitated and the peptide sequences expressed by
the cloned phage were determined. The cloned phage
expressed various different peptides, including those
5   shown in Table 2. Similarly, CX$_7$C (SEQ ID NO: 11) and
CX$_5$C (SEQ ID NO: 9) libraries were screened and breast
tumor homing peptides were identified (Table 2). These
results demonstrate that *in vivo* panning against a breast
tumor can identify tumor homing molecules.

10   **EXAMPLE VI**
           IN VIVO TARGETING OF A PHAGE EXPRESSING AN
                   AN RGD PEPTIDE TO A TUMOR

Human 435 breast carcinoma cells were
inoculated into the mammary fat pad of nude mice. When
15   the tumors attained a diameter of about 1 cm, phage
expressing a specific RGD-containing peptide were
administered to the tumor bearing mouse. Similar results
to those discussed below also were obtained with nude
mice bearing tumors formed by implantation of human
20   melanoma C8161 cells or by implantation of mouse B16
melanoma cells.

1 x 10^9 phage expressing the RGD-containing
peptide, CDCRGDCFC (SEQ ID NO: 1; see, Koivunen et al.,
supra, 1995) or control (insertless) phage were injected
25   intravenously (iv) into the mice and allowed to circulate
for 4 min. The mice then were snap frozen or perfused
through the heart while under anesthesia, and various
organs, including tumor, brain and kidney, were removed
and the phage present in the organs was quantitated (see
30   U.S. Patent No. 5,622,699; Pasqualini and Ruoslahti,
supra, 1996).

Approximately 2-3 times more phage expressing
the CDCRGDCFC (SEQ ID NO: 1) peptide were detected in the
breast tumor as compared to brain and kidney, indicating
the CDCRGDCFC (SEQ ID NO: 1; RGD phage) peptide resulted
5   in selective homing of the phage to the breast tumor. In
a parallel study, unselected phage, which express
various, diverse peptides, were injected into
tumor-bearing mice and various organs were examined for
the presence of phage. Far more phage were present in
10 kidney and, to a lesser extent, brain, as compared to the
tumor. Thus, the 80-fold more RGD-expressing phage than
unselected phage concentrated in the tumor. These
results indicate that phage expressing the RGD-containing
peptide home to a tumor, possibly due to the expression
15 of the $\alpha_v\beta_3$ integrin on blood vessels forming in the
tumor.

Specificity of the breast tumor homing peptide
was demonstrated by competition experiments, in which
coinjection of 500 µg free peptide, ACDCRGDCFCG (SEQ ID
20 NO: 16; see Pasqualini et al., *supra*, 1997) with the
phage expressing the tumor homing peptide reduced the
amount of phage in the tumor by about tenfold, whereas
coinjection with the inactive control peptide, GRGESP
(SEQ ID NO: 17) essentially had no effect. These results
25 demonstrate that phage displaying a peptide that can bind
to an integrin expressed on angiogenic vasculature can
selectively home *in vivo* to an organ or tissue such as a
tumor containing such vasculature.

92

EXAMPLE VII

IMMUNOHISTOLOGIC ANALYSIS OF TUMOR HOMING PEPTIDES

This example provides a method of identifying
the localization of tumor homing molecules by
5 immunohistologic examination.

Localization of phage expressing a tumor homing
peptide was identified by immunochemical methods in
histologic sections obtained either 5 min or 24 hr after
administration of phage expressing a tumor homing peptide
10 ("peptide-phage") to a tumor bearing mouse. For samples
obtained 5 min following administration of the
peptide-phage, mice were perfused with DMEM and various
organs, including the tumor, were removed and fixed in
Bouin's solution. For samples obtained at 24 hr, no
15 peptide-phage remains in the circulation and, therefore,
perfusion was not required. Histologic sections were
prepared and reacted with anti-M13 (phage) antibodies
(Pharmacia Biotech; see U.S. Patent No. 5,622,699;
Pasqualini and Ruoslahti, *supra*, 1996). Visualization of
20 the bound anti-M13 antibody was performed using a
peroxidase-conjugated second antibody (Sigma; St. Louis
MO) according to the manufacturer's instructions.

As discussed in Example VI, phage expressing
the tumor homing peptide, CDCRGDCFC (SEQ ID NO: 1; "RGD
25 phage), were administered intravenously to mice bearing
the breast tumor. In addition, the RGD phage were
administered to mice bearing a mouse melanoma or a human
Kaposi's sarcoma. Circulation of the phage was
terminated and mice were sacrificed as described above
30 and samples of the tumor and of skin adjacent to the
tumor, brain, kidney, lung and liver were collected.
Immunohistochemical staining for the phage showed

accumulation of the RGD phage in the blood vessels
present in the breast tumor as well as in the melanoma
and the Kaposi's sarcoma, whereas little or no staining
was observed in the control organs.

5   Similar experiments were performed using phage
expressing the tumor homing peptide, CNGRCVSGCAGRC (SEQ
ID NO: 3; "NGR phage"), which was identified by *in
vivo* panning against a tumor formed by the MDA-MB-435
breast carcinoma. In these experiments, NGR phage or
10 control phage, which do not express a peptide, were
administered to mice bearing tumors formed by the
MDA-MB-435 breast carcinoma or by a human SLK Kaposi's
sarcoma xenograft, then the mice were sacrificed as
described above and tumors were collected as well as
15 control organs, including brain, lymph node, kidney,
pancreas, uterus, mammary fat pad, lung, intestine, skin,
skeletal muscle, heart and epithelium of the renal
calices, bladder and ureter. Histological samples were
prepared and examined by immunostaining as described
20 above.

           In samples obtained from mice sacrificed 4 min
after administration of the NGR phage, immunostaining of
the vasculature of both the breast tumor and the Kaposi's
sarcoma was observed. Very little or no staining was
25 observed in the endothelium of the these tumors in mice
administered an insertless control phage. In the samples
obtained from mice sacrificed 24 hr after administration
of the NGR phage, staining of the tumor samples appeared
to have spread outside of the vessels, into the breast
30 tumor parenchyma and the Kaposi's sarcoma parenchyma.
Again, little or no staining was observed in samples
prepared from these tumors in mice administered the
insertless control phage. In addition, little or no

staining was observed in various control organs in mice
administered the NGR phage.

5   In other experiments, similar results were
obtained following administration of phage expressing the
NGR tumor homing peptides, NGRAHA (SEQ ID NO: 6) or
CVLNGRMEC (SEQ ID NO: 7), to tumor bearing mice. Also,
as discussed below, similar results were obtained using
phage expressing the GSL tumor homing peptide, CLSGSLSC
(SEQ ID NO: 4), which was identified by *in vivo* panning
10 of a melanoma (see Example VIII, below).

        These results demonstrate that tumor homing
peptides selectively home to tumors, particularly to the
vasculature in the tumors and that tumor homing peptides
identified, for example, by *in vivo* panning against a
15 breast carcinoma also selectively home to other tumors,
including Kaposi's sarcoma and melanoma. In addition,
these results demonstrate that immunohistochemical
analysis provides a convenient assay for identifying the
localization of phage expressing tumor homing peptides.

20   **EXAMPLE VIII**

          IDENTIFICATION OF TUMOR HOMING PEPTIDES
        BY *IN VIVO* PANNING AGAINST A MELANOMA TUMOR

        The general applicability of the *in vivo*
panning method to identify tumor homing peptides was
25 examined by performing *in vivo* panning against an
implanted mouse melanoma tumor.

        Mice bearing a melanoma were produced by
implantation of B16B15b mouse melanoma cells, which
produce highly vascularized tumors. B16B15b mouse
30 melanoma cells were injected subcutaneously into the

mammary fat pad of nude mice (2 months old) and tumors
were allowed to grow until the diameter was about 1 cm.
In vivo panning was performed as disclosed above.
Approximately 1 x 10^{12} transducing units of phage
5   expressing the CX$_5$C (SEQ ID NO: 9), CX$_6$C (SEQ ID NO: 10)
or CX$_7$C (SEQ ID NO: 11) library were injected, iv, and
allowed to circulate for 4 min. Mice then were snap
frozen in liquid nitrogen or perfused through the heart
while under anesthesia, tumor tissue and brain (control
10 organ) were removed, and phage were isolated as described
above. Three rounds of *in vivo* panning were performed.

TABLE 3		
PEPTIDES FROM PHAGE RECOVERED FROM MOUSE B16B15b MELANOMA		
CLSGSLSC (4*)	GICKDDWCQ (107)	TSCDPSLCE (108)
KGCGTRQCW (109)	YRCREVLCQ (110)	CWGTGLC (111)
WSCADRTCM (112)	AGCRLKSCA (113)	SRCKTGLCQ (114)
PICEVSRCW (115)	WTCRASWCS (116)	GRCLLMQCR (117)
TECDMSRCM (118)	ARCRVDPCV (119)	CIEGVLGGC (120)
CSVANSC (121)	CSSTMRC (122)	SIDSTTF (123)
GPSRVGG (124)	WWSGLEA (125)	LGTDVRQ (126)
LVGVRLL (127)	GRPGDIW (128)	TVWNPVG (129)
GLLLVVP (130)	FAATSAE (131)	WCCRQFN (132)
VGFGKAL (133)	DSSLRLP (134)	KLWCAMS (135)
SLVSFLG (136)	GSFAFLV (137)	IASVRWA (138)
TWGHLRA (139)	QYREGLV (140)	QSADRSV (141)
YMFWTSR (142)	LVRRWYL (143)	TARGSSR (144)
TTREKNL (145)	PKWLLFS (146)	LRTNVVH (147)
AVMGLAA (148)	VRNSLRN (149)	

* – numbers in parentheses indicate SEQ ID NO:.

30

The amino acid sequences were determined for
the inserts in 89 cloned phage recovered from the B16B15b

tumors. The peptides expressed by these phage were
represented by two predominant sequences, CLSGSLSC (SEQ
ID NO: 4; 52% of the clones sequenced) and WGTGLC (SEQ ID
NO: 18; 25% of the clones; see Table 3). Reinfection of
5 phage expressing one of the selected peptides resulted in
approximately three-fold enrichment of phage homing to
the tumor relative to brain.

        Localization of the phage expressing a tumor
homing peptide in the mouse organs also was examined by
10 immunohistochemical staining of the tumor and various
other tissues (see Example VII). In these experiments,
1×10^9 pfu of a control (insertless) phage or a phage
expressing the tumor homing peptide, CLSGSLSC (SEQ ID
NO: 4), were injected, iv, into tumor bearing mice and
15 allowed to circulate for 4 min.

        Immunostaining was evident in the melanoma
obtained from a mouse injected with phage expressing the
CLSGSLSC (SEQ ID NO: 4) tumor homing peptide. Staining
of the melanoma generally was localized to the blood
20 vessels within the tumor, although some staining also was
present in the tumor parenchyma. Essentially no staining
was observed in a tumor obtained from a mouse injected
with the insertless control phage or in skin or in kidney
samples obtained from mice injected with either phage.
25 However, immunostaining was detected in the liver
sinusoids and in spleen, indicating that phage can be
trapped nonspecifically in organs containing RES.

        Using similar methods, *in vivo* panning was
performed in mice bearing a SLK human Kaposi's sarcoma.
30 Tumor homing peptides were identified and are disclosed
in Table 4. Together, these results demonstrate that the
in vivo panning method is a generally applicable method

for screening a phage library to identify phage
expressing tumor homing peptides.

TABLE 4

PEPTIDES FROM PHAGE RECOVERED FROM HUMAN KAPOSI'S SARCOMA

5	TDCTPSRCT (150*)	SWCQFEKCL (151)	VPCRFKQCW (152)
	CTAMRNTDC (153)	CRESLKNC (154)	CMEMGVKC (155)
	VTCRSLMCQ (156)	CNNVGSYC (157)	CGTRVDHC (158)
	CISLDRSC (159)	CAMVSMED (160)	CYLGVSNC (161)
	CYLVNVDC (162)	CIRSAVSC (163)	LVCLPPSCE (164)
10	RHCFSQWCS (165)	FYCPGVGCR (166)	ISCAVDACL (167)
	EACEMAGCL (168)	PRCESQLCP (169)	RSCIKHQCP (170)
	QWCSRRWCT (171)	MFCRMRSCD (172)	GICKDLWCQ (173)
	NACESAICG (174)	APCGLLACI (175)	NRCRGVSCT (176)
	FPCEGKKCL (177)	ADCRQKPCL (178)	FGCVMASCR (179)
15	AGCINGLCG (180)	RSCAEPWCY (181)	DTCRALRCN (182)
	KGCGTRQCW (109)	GRCVDGGCT (183)	YRCIARECE (184)
	KRCSSSLCA (185)	ICLLAHCA (186)	QACPMLLCM (187)
	LDCLSELCS (188)	AGCRVESC (189)	HTCLVALCA (190)
	IYCPGQECE (191)	RLCSLYGCV (192)	RKCEVPGCQ (193)
20	EDCTSRFCS (194)	LECVVDSCR (195)	EICVDGLCV (196)
	RWCREKSCW (197)	FRCLERVCT (198)	RPCGDQACE (199)
	CNKTDGDEGVTC (15)		

* – numbers in parentheses indicate SEQ ID NO:.

EXAMPLE IX

25　　CHARACTERIZATION OF A CHIMERIC PEPTIDE COMPOSED OF A
　　　　　PROSTATE-HOMING PEPTIDE AND $_D$(KLAKLAK)$_2$

This example demonstrates that the chimeric
peptide SMSIARL-GG-$_D$(KLAKLAK)$_2$ can selectively induce
apoptosis in prostate tissue following systemic

administration and can prolong survival of animals with
experimental prostate cancer.

A. Isolation of prostate homing peptides

        An X_7 library was injected into mice, and
5   sequences isolated that were preferentially found in
prostate as compared to brain as described in
WO 99/46284. The prostate homing peptides SMSIARL (SEQ
ID NO: 207) and VSFLEYR (SEQ ID NO: 222) exhibited a
34-fold and 17-fold enrichment, respectively, in prostate
10 as compared to brain. Additional prostate homing
sequences identified by *in vivo* panning are shown in
Table 5.

	TABLE 5		
	PEPTIDES FROM PHAGE RECOVERED FROM PROSTATE		
EVQSAKW	(209)	KRVYVLG	(210)
GRLSVQV	(211)	WKPASLS	(212)
FAVRVVG	(213)	LVRPLEG	(214)
GFYRMLG	(215)	EGRPMVY	(216)
GSRSLGA	(217)	RVWQGDV	(218)
GDELLA	(219)	FVWLVGS	(220)
GSEPMFR	(221)	VSFLEYR	(222)
WHQPL	(223)	SMSIARL*	(207)
RGRWLAL*	(224)	QVEEFPC	(225)
LWLSGNW	(226)	GPMLSVM	(227)
WTFLERL	(228)	VLPGGQW	(229)
REVKES	(230)	RTPAAVM	(231)
GEWLGEC	(232)	PNPLMPL	(233)
SLWYLGA	(234)	YVGGWEL	(235)

Parentheses contain SEQ ID NO:.
* indicates sequences isolated more than once.

B. Prostate homing of prostate-homing peptide biotin
conjugates

Using *in vivo* screening of a heptapeptide phage
library, a prostate-homing peptide has been identified
5   that concentrates in the prostate 35-fold more than in
various other tissues. This phage displays the peptide
SMSIARL (SEQ ID NO: 207). Coinjecting synthetic SMSIARL
peptide (SEQ ID NO: 207) inhibited the prostate-selective
homing of phage bearing SMSIARL (SEQ ID NO: 207).
10 Furthermore, antibody staining of tissue sections showed
that SMSIARL (SEQ ID NO: 207) phage localized to prostate
tissue, but not to other tissues after an intravenous
injection of the phage into mice. Control phage also did
not accumulate in the prostate. SMSIARL (SEQ ID NO: 207)
15 phage also homed to rat prostate tissue.

As shown in Figure 6, the biotin-conjugated
SMSIARL (SEQ ID NO: 207) synthetic peptide was shown to
home to the prostate. Briefly, one mg of
biotin-conjugated prostate-homing peptide SMSIARL (SEQ ID
20 NO: 207), or biotin-labeled control peptide CARAC (SEQ ID
NO: 208), was injected intravenously into a mouse, which
was sacrificed 10 minutes later. The prostate and other
tissues were collected, sectioned and processed for
staining with avidin-peroxidase. Biotin staining was
25 found mostly in the lumen of the glands, rather than in
the vasculature, as soon as 10 minutes after systemic
injection of the conjugate. These results indicate that
the SMSIARL (SEQ ID NO: 207) peptide, as well as other
moieties attached to it such as phage or biotin,
30 translocates to the prostate epithelium and then into the
glandular lumen.

C. Induction of prostate-selective apoptosis

The ability of the SMSIARL peptide (SEQ ID NO: 207) to deliver a pro-apoptotic peptide, $_D$(KLAKLAK)$_2$, to the prostate was analyzed. Briefly,

5 SMSIARL-GG-$_D$(KLAKLAK)$_2$ chimeric peptide or control material was administered as a single dose of 250 μg peptide/mouse, and TUNEL staining was performed on tissues obtained 24 hours later. As shown in Figure 7, mice injected with the SMSIARL-GG-$_D$(KLAKLAK)$_2$ chimera

10 showed increased apoptosis in their prostates and, in particular, in capillary endothelium and the basal myoepithelial cells of prostate glands. There was no evidence of increased apoptosis in other tissues such as the testis, kidney or brain. Apoptosis was not observed

15 in negative control mice treated with 250 μg of an unconjugated mixture of SMSIARL (SEQ ID NO: 207) and $_D$(KLAKLAK)$_2$.

D. SMSIARL-GG-$_D$(KLAKLAK)$_2$ treatment of TRAMP mice

TRAMP mice develop prostate cancer under the

20 influence of a transgene as described in Gingrich et al., *supra,* 1996. SMSIARL-GG-$_D$(KLAKLAK)$_2$ chimeric peptide was assayed for the ability to suppress cancer development in the TRAMP mice. Treatment was initiated at 12 weeks of age with mice (10 per group) receiving

25 SMSIARL-GG-$_D$(KLAKLAK)$_2$ peptide or control peptide at 250 μg/dose every other week for a total of 10 doses. Four mice, which had no visible tumor, were eliminated from the SMSIARL-GG-$_D$(KLAKLAK)$_2$ group after dying within minutes after the injection. As shown in Figure 8,

30 SMSIARL-GG-$_D$(KLAKLAK)$_2$-treated mice survived longer than mice treated with controls, which were vehicle, $_D$(KLAKLAK)$_2$ peptide alone, or SMSIARL peptide (SEQ ID NO:

207) alone.   Thus, treatment of TRAMP mice with targeted
pro-apoptotic SMSIARL-GG-$_D$(KLAKLAK)$_2$ compound over a
period of several months resulted in an apparent increase
in survival of the treated mice relative to control mice.

5 E. Prostate-homing SMSIARL (SEQ ID NO: 207) phage bind to
human prostate vasculature

         A human prostate tissue section containing both
normal and cancerous tissue was overlaid with 10^9 TU
SMSIARL phage (SEQ ID NO: 207), and the binding of the
10 phage was detected with anti-phage antibody and
peroxidase staining. As shown in Figure 9, SMSIARL (SEQ
ID NO: 207) phage bind to the endothelium of human
prostate blood vessels (see panels a and b). No
endothelial staining was seen with phage that contain no
15 peptide insert (panel c). Furthermore, the SMSIARL (SEQ
ID NO: 207)-phage staining was inhibited when soluble
SMSIARL peptide SEQ ID NO: 207 was included in the
overlay at 0.3 mg/ml. The results shown in Figure 9
indicate that at least some tumors retain the receptor
20 for the homing peptide. Furthermore, peptide SEQ ID
NO: 207 can bind to vessels in intraprostatic cancer,
while blood vessels in several other human tissues were
not stained by the SMSIARL (SEQ ID NO: 207) phage.

         All journal article, reference, and patent
25 citations provided above, in parentheses or otherwise,
whether previously stated or not, are incorporated herein
by reference.

         Although the invention has been described with
reference to the examples above, it should be understood
30 that various modifications can be made without departing

102

from the spirit of the invention. Accordingly, the
invention is limited only by the following claims.

We claim:

1.   A chimeric prostate-homing pro-apoptotic peptide, comprising a prostate-homing peptide linked to an antimicrobial peptide,

  said chimeric peptide selectively internalized by prostate tissue and exhibiting high toxicity thereto, and

  said antimicrobial peptide having low mammalian cell toxicity when not linked to said prostate-homing peptide.

2.   The chimeric peptide of claim 1, wherein said prostate-homing peptide comprises the sequence SMSIARL (SEQ ID NO: 207), or a functionally equivalent sequence.

3.   The chimeric peptide of claim 1, wherein said antimicrobial peptide has an amphipathic α-helical structure.

4.   The chimeric peptide of claim 1, wherein said antimicrobial peptide comprises a sequence selected from the group consisting of:

    $(KLAKLAK)_2$ (SEQ ID NO: 200);

    $(KLAKKLA)_2$ (SEQ ID NO: 201);

    $(KAAKKAA)_2$ (SEQ ID NO: 202); and

    $(KLGKKLG)_3$ (SEQ ID NO: 203).

5.   The chimeric peptide of claim 1, wherein said antimicrobial peptide comprises the sequence $_D(KLAKLAK)_2$.

6.   The chimeric peptide of claim 5, comprising the sequence SMSIARL-GG-$_D(KLAKLAK)_2$.

104

7.   The chimeric peptide of claim 6, consisting of the sequence SMSIARL-GG-$_D$(KLAKLAK)$_2$.

8.   A method of directing an antimicrobial peptide *in vivo* to a prostate cancer, comprising administering the chimeric peptide of claim 1.

9.   The method of claim 8, wherein said prostate-homing peptide comprises the sequence SMSIARL (SEQ ID NO: 207), or a functionally equivalent sequence.

10.   The method of claim 8, wherein said antimicrobial peptide comprises the sequence $_D$(KLAKLAK)$_2$.

11.   The method of claim 10, wherein said chimeric peptide comprises the sequence SMSIARL-GG-$_D$(KLAKLAK)$_2$.

12.   The method of claim 11, wherein said chimeric peptide is SMSIARL-GG-$_D$(KLAKLAK)$_2$.

13.   A method of inducing selective toxicity *in vivo* in a prostate cancer, comprising administering the chimeric peptide of claim 1 to a subject having prostate cancer.

14.   The method of claim 13, wherein said prostate-homing peptide comprises the sequence SMSIARL (SEQ ID NO: 207), or a functionally equivalent sequence.

15.   The method of claim 13, wherein said antimicrobial peptide comprises the sequence $_D$(KLAKLAK)$_2$.

16.   The method of claim 15, wherein said chimeric peptide comprises the sequence SMSIARL-GG-$_D$(KLAKLAK)$_2$.

17.   The method of claim 16, wherein said chimeric peptide is SMSIARL-GG-$_D$(KLAKLAK)$_2$.
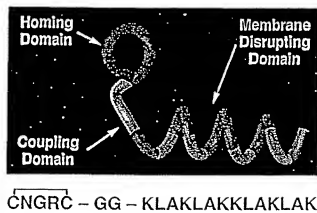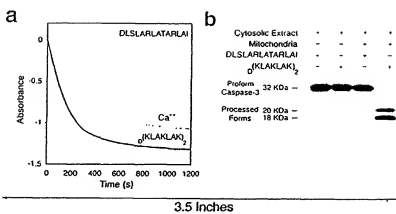
18.   A method of treating a patient having a prostate cancer, comprising administering the chimeric
5   peptide of claim 1 to said patient, whereby said chimeric peptide is selectively toxic to said tumor.

19.   The method of claim 18, wherein said prostate-homing peptide comprises the sequence SMSIARL (SEQ ID NO: 207), or a functionally equivalent sequence.

10   20.   The method of claim 18, wherein said antimicrobial peptide comprises the sequence $_D$(KLAKLAK)$_2$.

21.   The method of claim 20, wherein said chimeric peptide comprises the sequence SMSIARL-GG-$_D$(KLAKLAK)$_2$.

22.   The method of claim 21, wherein said chimeric
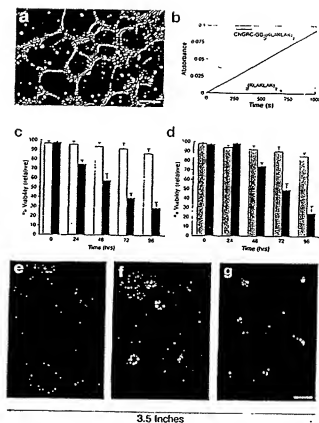15   peptide is SMSIARL-GG-$_D$(KLAKLAK)$_2$.

CNGRC – GG – KLAKLAKKLAKLAK
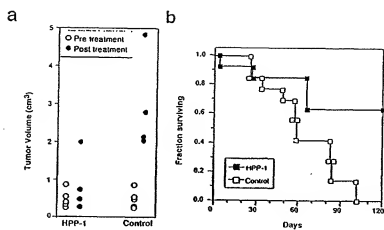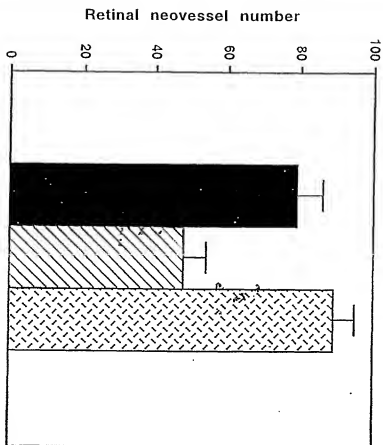
Figure 1

3.5 Inches

Figure 2

3.5 inches

Figure 3

a

b

Figure 4

Retinal neovessel number

Figure 5

Figure 6

Figure 7

OF DAYS

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- --○-- Vehicle
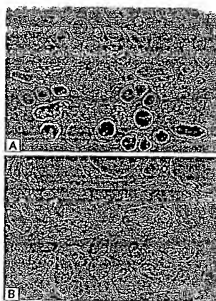- --□-- $_D$(KLAKLAK)$_2$
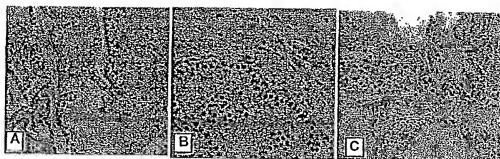- --■-- SMSIARL (SEQ ID NO:207)
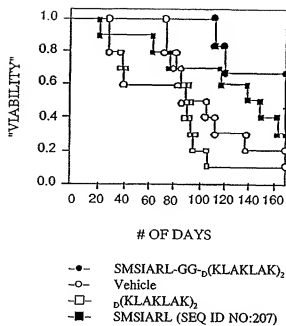
Figure 8

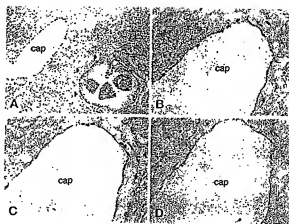Figure 9

SEQUENCE LISTING

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       Ruoslahti, Erkki
       Pasqualini, Renata
       Wadih, Arap
       Bredesen, Dale E.
       Ellerby, H. Michael

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A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   C07K14/47   A61K47/48   A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C07K   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, CHEM ABS Data, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1999 ELLERBY H MICHAEL ET AL: "Anti-cancer activity of targeted pro-apoptotic peptides." Database accession no. PREV199900484607 XP002164829 abstract & NATURE MEDICINE, vol. 5, no. 9, 1999, pages 1032-1038, ISSN: 1078-8956 ---/--	1-22

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 April 2001	30.04.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Döpfer, K-P

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.

Y	WO 99 46284 A (BURNHAM INST)
	16 September 1999 (1999-09-16)
	  claims 1-5
	  tables 1,7
	page 54, line 28 -page 55, line 3
	page 56, line 18 -page 57, line 3
	---- 	
		1-22

P,X	WO 00 42973 A (BURNHAM INST) 27 July 2000 (2000-07-27) the whole document ----	1-22
A	WO 99 13329 A (BURNHAM INST) 18 March 1999 (1999-03-18) claims 9-36 ----	1-22
A	WO 98 10795 A (BURNHAM INST) 19 March 1998 (1998-03-19) page 4, line 11 -page 5, line 26 claims ----	1-22
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1996 JAVADPOUR MARYAM M ET AL: "De novo antimicrobial peptides with low mammalian cell toxicity." Database accession no. PREV199699130909 XP002164830 abstract & JOURNAL OF MEDICINAL CHEMISTRY, vol. 39, no. 16, 1996, pages 3107-3113, ISSN: 0022-2623 ----	1,3-7
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1997 JAVADPOUR MARYAM M ET AL: "Self-assembly of designed antimicrobial peptides in solution and micelles." Database accession no. PREV199799701983 XP002164831 abstract & BIOCHEMISTRY, vol. 36, no. 31, 1997, pages 9540-9549, ISSN: 0006-2960 ----	1,3-7

INTERNATIONAL SEARCH REPORT

Box I	Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: —
   because they relate to subject matter not required to be searched by this Authority, namely:

   see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Continuation of Box I.1

Although claims 8-22 are directed to a method of treatment of the
human/animal body, the search has been carried out and based on the
alleged effects of the compound/composition.

Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by
therapy

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9946284	A	16-09-1999	US	6174687 B	16-01-2001
			AU	3078399 A	27-09-1999
			EP	1062232 A	27-12-2000
WO 0042973	A	27-07-2000	AU	3348600 A	07-08-2000
WO 9913329	A	18-03-1999	US	6180084 B	30-01-2001
			AU	9477398 A	29-03-1999
			EP	1015884 A	05-07-2000
WO 9810795	A	19-03-1998	AU	4412297 A	02-04-1998
			EP	0927045 A	07-07-1999
			JP	2001501600 T	06-02-2001